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# Remarques sur l'hématologie géographique

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Le concept d'hématologie géographique est relativement récent mais les faits qu il assemble ne sont pas tous neufs. On sait depuis longtemps que l anatomie, la physiologie, la pathologie du sang dépendent pour une large part des peuples et des races, du sol, de l'air des climats, des coutumes alimentaires, des infections, des parasitoses particulières à certaines régions. On a, en ces dernières années, souligné la place privilégiée que tient l'hématologie parmi les disciplines biologiques. Les caractères sanguins sont assurément ceux dont les variations géographiques sont le plus aisément appréciées et mesurées. Ainsi l importance de l'hématologie géographique est très grande. Le diagnostic d un désordre sanguin, d une anémie, d une éosinophilie est parfois d emblée orienté, voire assuré par la géographie. Surtout l hématologie géographique permet, dans des conditions très favorables, l analyse des rôles respectifs des caractères innés et de l'environnement, l étude des changements et des adaptations. Enfin, offrant des modèles d'hémopathies dont la cause est connue, elle vient faciliter les enquêtes étiologiques, elle nous munit de modes de raisonnement, de méthodes d enquêtes qui devraient, appliquées aux hémopathies dont la cause nous échappe encore, permettre de nouveaux progrès.

Laissant de côté les aspects pratiques et cliniques, nous voudrions dans ce rapport souligner la valeur exemplaire de l'hématologie géographique, l abord original qu elle permet de certains problèmes fondamentaux.

## *I Hématologie ethnico-sociale*

L influence qu exercent sur la santé de l'homme et plus particulièrement sur son sang le climat physique et ses variations est bien définie. L influence des progrès de la civilisation, de la transformation du milieu naturel du changement des structures sociales est moins bien connue. Elle mérite d être examinée. Cet examen per

met d'isoler une hématologie ethnico-sociale. A chacun des grands types d organisation culturelle et sociale, à chacun des grands paliers évolutifs, correspond une pathologie sanguine singulière.

1 *Civilisation primitive* Les sociétés primitives pratiquant soit la cueillette et la chasse, soit la polyculture de subsistance, soit les deux, sont étroitement liées à leur milieu naturel. Quelques peu plades vivent encore de nos jours sur le mode archaïque. On les rencontre sur presque tous les continents Aetus des Philippines, Semangs et Sakais de Malaisie, Australiens aborigènes de la terre d Arnhem et de la presqu'île d'York, Chenchus de l Inde méridionale, Veddas de Ceylan indigènes des îles Andoman, Bushmen et Pygmées d Afrique Centrale et du Sud, Guyakis et Fuegicos d Amérique du Sud. Leur hématologie a été peu étudiée. On peut cependant noter les deux traits suivants

a) Les carences alimentaires sont rares. Les primitifs jouissent d une alimentation équilibrée, subissant tout au plus des restrictions saisonnières. Chez eux point d anémie de carence. L étude ostéologique des restes fossiles du paléolithique supérieur n a jamais révélé de signes carentiels.

b) Ces sociétés primitives sont en revanche atteintes par les grandes maladies sanguines d agression biologique. L'homme primitif vit sans aucune protection dans un milieu auquel il est rigoureusement lié. Simple élément d une biocénose complexe, il vient s inclure dans une série de cycles parasitaires ou il joue le rôle inévitable d'hôte naturel. Pour une zône donnée l ensemble du groupe humain est atteint. Tous les individus présentent les mêmes déviations sanguines en général d origine parasitaire et souvent assez bien supportées.

2 *Sociétés plus évoluées Monocultures de subsistance.* Les sociétés plus évoluées vouées à la monoculture sont dans leur début étroitement liées au milieu naturel (sociétés de type tropical) Aussi connaissent-elles encore de nombreux syndromes d agression biologique. Mais ici l'hématologie carentielle prend très vite une importance de premier plan à mesure que la technique libère l'homme du milieu biologique (prophylaxie) et que l économie l'intègre à un milieu social en déséquilibre.

On sait maintenant qu une modification de la nature du climat biologico-social peut changer le type hématologique de la population indigène.

En zône impaludée l assèchement des marais et leur remplacement par les forêts ou les cultures vivrières suppriment l ané

mie palustre et favorisent l accroissement de la courbe démogra phique.

On peut penser qu à la longue la disparition du plasmodium entraînera celle des hémoglobinoses et les déficiences en glucose-6-phosphate déhydrogénase qui, n ayant plus de rôle protecteur à jouer sont devenues des caractères défavorables.

L irrigation et la mise en valeur de certaines zônes désertiques ont souvent entraîné, en l absence d un contrôle rigoureux, l'extension de la schisostomose à des populations saines. On a pu dire dans ces cas que «l éosinophilie suivait l eau»

L'inégalité des conditions économiques explique l'inégale ré partition des anémies carentielles dans un groupe humain donné. Mais dans le monde actuel où, selon les statistiques récentes, la sous-nutrition totale ou partielle atteint les deux tiers de l'humanité, l importance des anémies carentielles doit être fortement soulignée.

Le remaniement brutal des structures agricoles traditionnelles, son remplacement par des types de culture de commercialisation ont créé de nouvelles formes de carences.

Le lien est habituel mais non constant entre la condition économique et l'anémie de carence. D autres facteurs religieux, sociaux, peuvent jouer Nous citerons deux exemples qui soulignent la valeur du concept d'hématologie sociale.

En Inde normalement, les anémies carentielles sont rares chez les parias qui, ayant le droit de consommer de la viande, ont, quoique pauvres, un régime alimentaire équilibré. Ces anémies sont au contraire fréquentes chez les Hindouistes, astreints à la seule nourriture végétale, où le riz tient une place variable suivant les saisons et les années, et où le lait, denrée de luxe pour beaucoup, est limité au rôle de condiment. Seuls les Hindouistes de la montagne, abondamment pourvus de lait, ne présentent guère de syndromes hématologiques carentiels. Leur état de santé, très satisfaisant dans l ensemble, contraste durement avec celui des mêmes castes de la plaine encombrées d infirmes et d'incurables.

Un deuxième exemple, assez démonstratif, nous est fourni par l apparition du syndrome de Kwashiorkor chez les enfants du Nigé ria. Le Kawshiorkor et l anémie macrocytaire qui l accompagne sont assez fréquents chez les enfants de familles indigènes qui, attirées par des salaires plus élevés, sont allées habiter la ville.

Ce nouveau mode de vie implique généralement l adoption de la monogamie et la réduction de la durée de l'allaitement maternel

la mère étant obligée de faire face aux travaux du ménage et parfois même d accepter un travail rémunéré. Ayant un pouvoir d achat très faible, obligés de faire vivre une famille souvent nombreuse, ces nouveaux citadins sont le plus souvent condamnés à un régime végétarien assez strict qui, chez le jeune enfant, trop tôt sevré, est insuffisant en matière protéique.

Dans la brousse, au contraire, l anémie macrocytaire carentielle est beaucoup plus rare l allaitement maternel se poursuit assez longtemps souvent jusqu à l âge de 2 ans, ce qui est facilité d ailleurs par la polygamie tribale. Quand l enfant est sevré, il est déjà capable de marcher et va à la recherche de chenilles, de termites et d autres insectes dont il se nourrit à longueur de journée. Cette «récolte» lui assure un apport protéique suffisant. Dans le cas des Nigériens ce sont les tribus de brousse qui présenteront le moins de signes de carence.

Ce phénomène ne paraît d ailleurs pas limité au Nigéria. Les rapports les plus récents indiquent que les cas de kwashiorkor sont en augmentation dans divers centres africains, tels que Nairobi, Kampala, Johannesburg etc.. La même progression vient d être signalée dans d autres agglomérations en dehors de l Afrique Noire en Indonésie, autour de Djakarta et de Macassar dans des villes japonaises du Nord du Hondo, dans l Inde autour de Madras, en Egypte, en Israël, etc..

De plus, l application des techniques industrielles à la monoculture intensive peut faire apparaître dans ce type de civilisation les premiers syndromes hématologiques liés aux toxiques.

3 *Sociétés modernes.* L intervention croissante des poisons du sang est en effet l un des caractères fondamentaux de l'hématologie des sociétés modernes industrielles. Substances menaçant seulement certaines catégories de travailleurs comme le benzène, médicaments menaçant les malades, ceux qui se croient malades ou les sujets sains victimes d une sollicitude prophylactique, insecticides et engrais menaçant les populations rurales, substances de synthèse employées pour nourrir vêtir porter tous les hommes la liste est longue des produits assurément ou éventuellement nocifs pour le sang Liste à laquelle il faut joindre les radiations dans leur emploi industriel. Le sang de l'homme des sociétés modernes est dangereusement exposé et une pathologie sanguine nouvelle, particulière à cette société se développe de nos jours.

Cette hématologie liée aux toxiques s'intrique et s'associe par fois à l'hématologie carentielle des stades précédents. La population des faubourgs industriels d Asie, d Afrique, d Amérique du Sud est encore exposée aux deux dangers, celui de l insuffisance alimentaire, celui des poisons industriels.

Cette description est délibérément schématique. De nombreux stades de transition existent entre les types divers d organisation que nous avons tenté d isoler

*II La valeur adaptative de certains caractères sanguins héréditaires*

Les caractères sanguins ont depuis longtemps été classés en deux catégories héréditaires et acquis.

*Les caractères sanguins héréditaires* définissent l hémotype. Ils furent longtemps considérés comme étant sous le seul contrôle génétique et échappant de manière totale aux conditions de milieu. Ce sont les groupes érythrocytaires, les protéines sériques spécifiques, les types hémoglobiniques, les enzymes cellulaires ou plasmatiques.

On sait maintenant que ces caractères ne sont pas neutres, mais possèdent, au moins pour certains d entre eux, une valeur adaptative.

Si chez nous le fait d appartenir au groupe A, B ou O ne confère aucun avantage évident, dans les pays où sévit le kyste hydatique en forte endémie (Irlande, Argentine) les individus O ou A semblent mieux résister à cette parasitose que les sujets B ou AB (Nunos Baratta)

Dans les régions d endémie pesteuse ou variolique, la peste paraît être plus fréquente et plus grave chez les sujets du groupe O et la variole chez ceux du groupe A (HELMBOLD et BOGEL, PETTENKOFFER)

Un autre exemple est donné par l'étude de la répartition des hémoglobines anormales. En 1953 ALLISON a démontré que *Plasmodium falciparum* vivait mal dans les hématies porteuses d'hémoglobines S et peut-être aussi dans celles qui portaient d autres hémoglobines telles que C, D ou E. Ces hémoglobines *représentent le type* d une mutation défavorable, au moins à l état hétérozygote, dans les régions fortement impaludées. Ce processus sélectif doit expliquer au moins en partie, la persistance d une haute fréquence d'hémoglobines anormales dans certaines zônes du globe.

Dans ces zônes à sévère endémie malarique, la présence d un pourcentage élevé d'hémoglobines anormales constitue un vérita

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tence de formes paludéennes graves chez des sujets enzymopéniques et, en Algérie, Suaudeau trouve un taux de déficience relativement modéré (2 %) dans la Mitidja, qui était fortement impaludée jusqu'à une date récente, et un taux plus élevé pour la grande Kabylie (3,35 %) où le paludisme fut sans doute moins répandu. Ici peut être faudrait-il invoquer l'importance de l'endogamie, beaucoup de populations de grande Kabylie constituant, depuis des siècles, de véritables isolats biologiques.

Au Liban, par contre, Ruffié et Taleb ont signalé que, parmi les ethnies numériquement importantes, seuls les Druzes habitant la montagne ne présentaient pas de déficience.

Il n'est donc pas possible de connaître encore, en toute certitude, le rôle exact que joue l'endémie palustre dans la persistance de l'enzymopénie. Si, comme il est assez vraisemblable, la persistance de la mutation enzymopénique à un taux élevé dans certaines zônes est sous le contrôle d'un phénomène de polymorphisme équilibré, des facteurs autres que l'infection plasmodiale ont pu intervenir ailleurs, au rang desquels on doit sans doute compter les conditions alimentaires. On peut penser en effet que, en dehors de toute agression toxique (médicamenteuse ou nutritionnelle), la mutation enzymopénique constitue un caractère indifférent sur le plan de la sélection.

Dans ce cas, la déficience ne constitue plus qu'un facteur racial neutre qui pourra atteindre une fréquence élevée dans des populations vivant en condition d'endogamie plus ou moins stricte. C'est peut-être le cas de certains foyers de grande Kabylie.

L'étude de certaines populations montagnardes apporte d'autres informations.

A l'heure actuelle, le peuplement des massifs montagneux tempérés apparaît comme fait d'isolats, dans lesquels l'hémotypologie permet de retrouver parfois les apports de populations successives.

Mais il est possible qu'ici encore se produise un processus de différenciation biologique de chaque groupe, que met bien en évidence l'étude des caractères sanguins. Le groupe de Walser en Suisse est assez évocateur. Les Walser quittèrent l'Oberland Bernois

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Mise à part, peut-être la forme B – de déficience totale, due à une mutation du gène de structure, rare, rencontrée seulement en Europe continentale et qui entretient une anémie hémolytique clinique permanente. Sa rareté même peut être expliquée par un processus de sélection naturelle.

au XIIème siècle pour aller vivre sur les hauts plateaux. Après sept siècles d isolement en montagne, ils ont conservé leur pureté ethnique (mœurs, langue allemande archaïque) Toutefois, en ce qui concerne leur type hématologique, deux constatations ont été faites

1 Les Walser diffèrent aujourd hui de manière hautement significative de la population parente dont ils se sont détachés il y a 700 ans, pour aller vivre dans les hauteurs (on trouve en particulier dans certains groupes Walser le plus fort pourcentage de sujets O Rh rencontrés en Europe)

2 Les Walser forment à l heure actuelle un certain nombre de groupes montagnards relativement isolés il existe souvent, entre ces groupes, des différences hémotypologiques nettes.

L isolat montagnard a donc eu une action diversifiante sur l évolution hématologique de ces populations qui devaient être initialement homogènes.

*La deuxième série de facteurs étudiés par l'hématologiste* correspond à des caractères généralement considérés comme acquis. La majorité porte sur des variations intéressant des éléments figurés du sang (éosinophilie, syndromes anémiques ou polyglobuliques, etc. ) En fait, cette distinction paraît moins rigoureuse qu on ne le pensait naguère.

La formule sanguine normale est, sans aucun doute, sous contrôle génétique. C'est ce qui assure sa fixité, pour les individus d une même espèce vivant dans un même milieu.

Cette formule peut varier sous l effet des multiples modifications de l environnement (agression bactérienne, parasitaire, virale, modifications climatiques, alimentaires, etc...)

Mais ces variations ne sont pas les mêmes pour tous les individus et pour toutes les races soumis à un stimulus identique ce qui donne à penser que l aptitude à varier dans tel ou tel sens est, elle aussi, sous contrôle génétique.

Ces variations acquises peuvent avoir une valeur adaptative. Tous les individus ne sont pas capables de s adapter avec la même efficacité à une modification péristasique ou à une agression pathogène.

Une helminthiase avec forte éosinophilie est parfois bien supportée chez l indigène de la grande forêt qui «accepte» son agresseur plus en symbionte qu en parasite. La même parasitose chez le sujet neuf peut entraîner les troubles les plus graves. Ici encore on peut admettre que l aptitude réactionnelle de l autochtone n est que

le résultat d'une sélection naturelle qui s'est exercée au cours de nombreuses générations.

Quoi qu'il en soit, ce type de caractère acquis paraît aujourd'hui étroitement conditionné par le milieu, pris dans son sens le plus large, tel que nous l'avons défini précédemment. A ce point de vue, l'exemple des anémies est hautement démonstratif.

1. Dans les sociétés peu développées (qui prédominent largement dans la zone intertropicale) les grands syndromes anémiques sont liés le plus souvent à des agressions biologiques. L'homme est soumis à la dure loi du milieu où il s'insère comme élément, parmi tant d'autres, d'une biocénose originale.

2. Les grandes anémies hémolytiques reconnaissent le paludisme, surtout en zone africaine ou asiatique, bien plus rarement en Amérique où le rôle de destructeur d'hématies est dévolu aux *Bartonella*.

Dans les mêmes régions, de fréquentes anémies hypochromes suivent les helminthiases qui saignent : ancylostomoses dans tout le monde tropical « débordant » parfois sur des foyers tempérés, toujours localisés, mais où se crée une écologie favorable (mines, chantiers de tunnels), foyers souvent entretenus par l'apport d'une main d'œuvre de couleur fortement parasitée; schistosomoses dont les principaux agents sont : *Schistosoma haematobium*, responsable de l'antique hématurie d'Egypte et qui, suivant les caravanes historiques, a depuis longtemps migré hors du foyer du Nil pour envahir le désert saharien, le Maghreb, le sud de l'Espagne et du Portugal, l'Afrique Equatorial à l'ouest, tout le Proche-Orient à l'Est ; *Schistosoma mansoni*, à localisation intestinale qui se superpose en foyers irréguliers dans toute la zone de *Schistosoma haematobium*, mais a en outre envahi l'Amérique du Sud à la faveur de la traite des esclaves noirs.

Dans les provinces Nord-Est du Brésil, *Schistosoma mansoni* est le grand responsable des anémies avec splénomégalies rencontrées chez l'enfant.

Citons enfin le troisième agent, le plus redoutable, *Schistosoma japonicum*, propre à l'Extrême-Orient, largement répandu de la Birmanie au Japon, en passant par toute la Chine du Sud, et qui est l'un des principaux « auteurs » de la pathologie des rizières.

3. Séparant l'homme du milieu naturel, le progrès social a entraîné, sans nul doute, la régression de ce type d'hémopathies. La schistosomose semble, depuis un siècle ou deux, avoir à peu près complètement disparu d'Europe. Partout, en zone tempérée, le paludisme a régressé et se localise aujourd'hui (largement il est vrai) dans la zone tropicale où se rencontrent les conditions climatiques les plus favorables à la réalisation du cycle plasmodial et où vivent des groupes humains souvent denses et toujours pauvres qui forment d'excellents réservoirs de virus.

Ici au contraire apparaissent les anémies carentielles, surtout dans les zones surpeuplées, où la courbe de production agricole ne suit pas, il s'en faut de loin, la courbe démographique. Le cas du syndrome de Kwashiorkor évoqué plus haut, est démonstratif. L'évolution de nombreux pays sous-développés est telle que « l'hématologie de carence » risque, longtemps encore, d'occuper l'une des premières places de la pathologie.

4. Dans les populations les plus évoluées, les syndromes d'agression biologique ou carentiels s'atténuent. L'homme est « protégé » par l'organisation sociale et la technique moderne; il vit dans ce nouveau milieu artificiel, qui l'isole favorablement de toute agression naturelle.

Le développement économique éloigne le spectre de la faim, réfugié pour un temps dans les couches les plus défavorisées de la société, dont l'importance s'amenuise.

Mais l'espèce humaine va payer un tribut à ce progrès : l'utilisation parfois abusive des produits de synthèse en agriculture, ou en thérapeutique, le rejet quotidien de tonnes de produits de déchets, souvent toxiques, dans les eaux ou l'atmosphère de la grande cité moderne, l'augmentation, isolée pour le moment, de la radioactivité, viennent jouer un rôle sans doute grandissant dans la pathologie hématologique.

Il agit ici d'une étiologie fine qui nous échappe encore le plus souvent. Les accidents du benzolisme ou les hémopathies des radiologistes n en constituent que des cas extrêmes, en quelque sorte caricaturaux. L évolution de la technique est telle que l'on peut craindre de voir le chapitre des hémopathies d origine toxique prendre une importance croissante dans les groupes humains les plus évolués.

### *III Les modèles étiologiques*

Une des faiblesses de l hématologie est l insuffisance des con naissances étiologiques. L étude de désordres sanguins, limitée à une région définie, survenant pendant une saison définie, nous dote de modèles dont certains ont très probablement une valeur générale.

*Fièvre hémorragique thaie* Il en est ainsi de la fièvre hémorragique thaie. L étiologie des purpuras thrombopéniques d Europe et d Amérique demeure confuse. La relation chronologique notée dans quelques cas avec des maladies virales, rubéole, oreillons, varicelle, n apporte pas la preuve du rôle étiologique de ces affections. Mais la fièvre hémorragique thaie, observée en Asie du Sud Est et surtout en Thaïlande et aux Philippines, atteignant surtout les petits enfants, se présente comme un purpura thrombopénique aigu assuré ment du à un virus Endémique, subissant des exacerbations épidé miques en automne, elle est due à un virus du groupe de la dengue, transmis par *Aedes aegypti*. Dans le tableau clinique se mêlent les signes de grande infection virale avec état ataxo-adynamique. col lapsus, et les hémorragies. La thrombopénie est remarquable par sa brièveté, remarquable aussi par son mécanisme. Elle parait bien liée à une mégacaryocytopénie précoce, également passagère et reconnue seulement par des ponctions médullaires faites au tout début de l évolution.

Ainsi la fièvre hémorragique thaie est doublement exemplaire elle est probablement la première thrombopénie dont l origine vi- rale soit assurée elle est aussi chez l'homme le premier exemple d un désordre médullaire du à un virus. La fréquente localisation de certains virus murins dans les mégacaryocytes accroît encore l in térêt suscité par la fièvre hémorragique thaie.

*La maladie de Burkitt*. La même valeur exemplaire peut être re connue à la maladie de Burkitt. Cet hématosarcome africain atteint surtout les enfants et est observé dans des régions où la leucémie ai guë des petits enfants est exceptionnelle.

Les safaris de Burkitt ont précisé la distribution géographique de la maladie et reconnu le rôle de deux facteurs d environnement la température (la tumeur de Burkitt n est observée que dans les

zônes dont la température moyenne est supérieure à 17 C) l'humidité point de tumeur dans les régions désertiques, nombreuses tumeurs là où les pluies sont abondantes. Le rôle de l humidité s explique probablement par l'influence qu elle exerce sur la végétation. Les interventions d un arthropode vecteur et d un virus sont dès lors sérieusement soupçonnées. Soupçonnées mais non encore prouvées. Les recherches en cours n ont pas encore donné de résultats formels. Mais il est très raisonnable d escompter et la démonstration de l origine virale et la valeur exemplaire de cette démonstration. Le sarcome de Burkitt serait la première tumeur maligne humaine dont l origine virale serait reconnue. On pourrait le tenir alors comme un de ces accidents naturels dont l étude révèle soudain de grandes vérités.

*Inégalité de la répartition géographique des leucémies*

Rien n illustre mieux l intérêt et aussi les difficultés de cette hématologie géographique que l étude de la répartition géographique des leucémies.

Les données que fournit cette étude sont les suivantes

1 La fréquence générale des leucémies varie beaucoup selon les nations. La fréquence des leucémies (au moins pour l'hémisphère Nord) est plus élevée pour les pays septentrionaux. Les variations sont surtout nettes pour les sujets les plus âgés.

2. Dans une nation donnée la fréquence des leucémies varie d une province, d une région à une autre.

3 Les diverses variétés sont inégalement réparties. Le fait le plus remarquable étant l'extrême rareté des leucémies lymphoïdes chroniques en Extrême Orient.

Des études en cours semblent montrer que la fréquence des leucémies lymphoïdes chroniques est beaucoup plus élevée chez les Japonais transplantés aux Etats-Unis. Ainsi seront mis en évidence – si ces données sont confirmées – le rôle des facteurs d environnement.

D'autres rapprochements seront peut-être féconds raison de la rareté des leucémies lymphoïdes chroniques des sujets jeunes, rapports éventuels entre les épizooties des leucémies bovines et les leucémies humaines.

Ainsi conçue, l hématologie humaine est, on le voit, étroitement liée au milieu. Si le sang reflète à coup sûr les caractères raciaux les plus fidèles, il demeure aussi le témoin rigoureux des agressions environnantes. Caractères génétiques et facteurs mésologiques

réagissent sans cesse les uns sur les autres. C'est dans la mesure où l être vivant demeure «adaptable» à une situation donnée qu il peut survivre et assurer une descendance. L'hématologie géographique est un fait d expérience qui s'inscrit maintenant dans la perspective des lois de la génétique et de l écologie c est un cas particulier mais combien démonstratif, de l interaction de l être vivant et de son milieu qui aboutit à «l adaptation» en dehors de laquelle le groupe est irrémédiablement condamné.


## *Résumé*

Après avoir rappelé l'importance du concept d'hématologie géographique, les auteurs étudient

1 L'hématologie ethnico-sociale à chacun des grands types d'organisation culturelle et sociale correspond une pathologie sanguine particulière (hématologie parasitaire des sociétés primitives, carentielle des monocultures de subsistance, toxique des sociétés modernes)

2 La valeur adaptative de certains caractères sanguins héréditaires (hémoglobines, déficits enzymatiques)

3 Certains modèles étiologiques fournis par l'hématologie géographique (fièvre hémorragique thaïe, maladie de Burkitt, géographie des leucémies.

## *Summary*

After recalling the importance of the concept of geographical haematology' the authors study

1 Ethnosocial aspects of haematology: each of the major types of cultural and social organization has typical haematopathology (parasitic blood diseases in primitive societies, deficiencies in single-crop subsistence cultures, intoxications in modern society)

2. The adaptation value of certain hereditary characteristics (haemoglobin types, enzyme deficiencies)

3. Some model etiological features emerging from study of haematology on geographical basis (Thai haemorrhagic fever Burkitt's disease, distribution of leukaemias)

## *Zusammenfassung*

Nach einem Hinweis auf die Wichtigkeit der geographischen Hämatologie werden besprochen

1 die ethnisch-soziale Hämatologie: jedem der großen Typen einer kulturellen und sozialen Organisation entspricht eine besondere Blutpathologie (parasitäre Krankheiten primitiver Völker Mangelerkrankungen bei einseitiger Ernährung, toxische Schädigungen der modernen Gesellschaft);

2. die Bedeutung der Anpassung bei gewissen vererbten Eigenschaften des Blutes (Hämoglobinanomalien, Enzymdefekte)

3 gewisse aetiologische Modelle der geographischen Hämatologie (hämorrhagisches Fieber Burkitt'sche Krankheit, geographische Verteilung der Leukämien)


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# Comparative Haematology of Laboratory Animals (Selected Aspects)

W LAWKOWICZ and F ZERSKI

It is a very difficult task to present such a vast and mostly unexplored field. Facts are scarce and there are many questions to be raised. Because of this, only selected aspects of comparative haematology of laboratory animals, especially those related to selection of material for experimental research according to species specificity will be discussed. We personally encountered numerous difficulties in past work in this respect. Discrepancies of results obtained by authors working on the same problem on different animals are only too many It is our feeling that the aspect of species specificity of haemopoietic reactions has sometimes a tendency to be overlooked.

Only general outlines of the whole complicated problem may be considered in the restricted space alloted to us. In certain instances tentative explanations of experimental discrepancies will be put forward, more often only questions may be posed and doubts expressed.

As morphology still remains the basis of our science, it is natural to begin with pointing out certain morphological differences between the blood cells of laboratory animals, expressing probably some underlying biochemical and/or functional differences.

Examining the peripheral blood of various animals one has to expect differences in haemoglobin levels, red cell counts and erythrocyte size, which may be explained by metabolic differences. It should be noticed here that WINTROBE's law (1) of constancy of the mean haemoglobin concentration in one red cell of about 30 % was demonstrated to be valid for hitherto examined mammals. Another side of the question present individual and temporal variations in red cell counts among representatives of the same species and of the same sex.

| Species | Cat Dog | Guinea-pig Rabbit | Rat, Mouse Hamster |
| --- | --- | --- | --- |
| Main blood reservoir | Spleen | Splanchnic vessel | on |
| Haematocrit | Labil | Labil | Nearly constant |

*Fig 1* Relationship between blood reservoirs and hematocrit lability

The red cell count and the haematocrit of mice, rats and hamsters shows a relative constancy in guinea-pigs and rabbits it is more labile, in cats and dogs surprisingly divergent results may be obtained. These phenomena may be correlated with the efficiency of blood reservoirs (Fig 1) Cats and dogs have a predominantly reservoir like spleen (2) the remaining species a predominantly metabolic one (3) but the splanchnic vessels of guinea pigs and rabbits are said to function as an efficient blood reservoir

It follows therefore that the erythrocytic response to acute blood loss, acute hypoxia, emotional and physical stress (n.b. during inconsiderate blood withdrawal) will be dependent on the species examined and no generally valid conclusions concerning the quantitative course of such a reaction may be drawn.

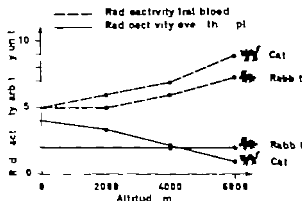



*Fig* Release of blood from the spleen of cat and lack of such phenomenon in rabbit during acute high altitude hypoxia.

This may be illustrated by the following experiment. Cats and rabbits were reinjected with their own red cells tagged with ⁵¹Cr and subjected to high altitude hypoxia the haematocrit, blood radioactivity and radioactivity over the spleen (using external collimated scintillation counters) being registered. The results as presented in Fig 2 illustrate the importance of the spleen in the mechanism of adaptative erythrocytosis in acute hypoxia in cats, as opposed to the lack of such a role in rabbits.

Species specificity is also reflected in the differential count. In spite of a varying degree of lability there exists a tendency to a granulocytic or lymphocytic blood picture dependent on the species (Fig 3) No explanation of this phenomenon has been found or even, as far as we know sought for (4)

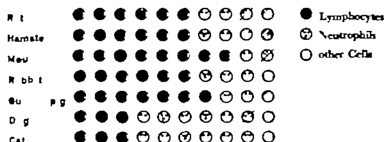



*Fig* 3. Mean differential counts in laboratory animals ( rounded)

Suggestions have been made that a correlation between the number of lymphocytes and blood proteins may exist No sufficient data are available to prove or to disprove this hypothesis. Nevertheless it is interesting to note that differences in serum protein levels and electrophoretic patterns among various laboratory animals (Fig 4 5 6) were demonstrated by our co-workers as well as by numerous other authors.

Even more perplexing are the morphologic differences among the granulocytes of various laboratory animals. The predominant type of these cells—the specific granulocytes corresponding to human neutrophils—differ in the stain affinity of cytoplasmic granulations. Three kinds of these may be distinguished (Fig 7) neutrophilic, pseudo-eosinophilic and chromophobic ones.

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Drs J Swierkowicz, M. Kraj J Zielinski, Z. Majewska to whom sincere thanks are expressed for kindly placing these data at our disposal.

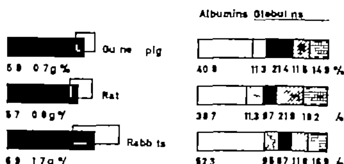



*Fig 4* *Serum protein level (g %) and electrophoretic patterns (%) in guinea-pigs, rats, rabbits.*

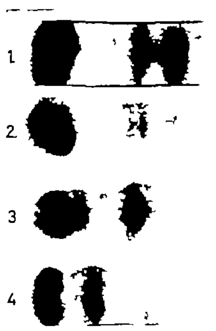



*Fig 5* Paper electrophorogramms 1 human serum, 2 rabbit serum, 3 guinea-pig serum, 4 rat serum

The shapes of granulocyte nuclei present a pleasant diversity beginning with the ring forms characteristic for mice, rats and hamsters and ending on the multisegmented cells predominant in rabbit blood (Fig 8) Investigations on rat bone marrow led to the hypothesis that the shape of the granulocytic nucleus may be dependent on the arrangement of chromosomes during metaphase and perhaps related to loss of chromosomes during consecutive

meiosis-like divisions of maturing granulocytes (5, 6). The obvious implication is that the shape of the nucleus would indicate differences in genetic information contained in the cells. According to ROHR's concept (7) of granulocyte maturation and the observa-

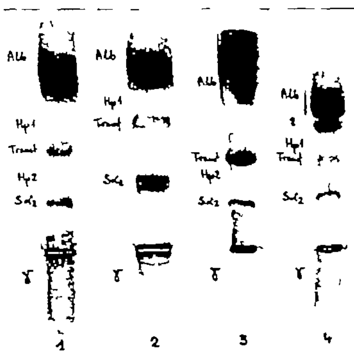



*Fig. 6.* As on the preceding figure haptoglobins, transferrin, S alpha 2 marked. Note the unidentified fraction in rat serum

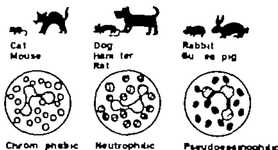



*Fig. 7.* Comparison of stain affinity of cytoplasmic granulations in specific granulocytes of laboratory animals.



*Fig. 11* Transformed lymphocytes in phytohaemagglutinin *in vitro* culture

a *suitable medium without addition of phytohaemagglutinin*, a similar transformation occurs after 6–8 days in a small percentage of cells. According to personal experiences, rabbit lymphocytes are noteworthy for their ability to show such an autotransformation of a remarkably high degree i.e. in 40–60 The significance of these experiments is difficult to assess, nevertheless it seems that a promising approach for claryfying species differences in lymphocyte reactivity opens here.

Another point worth noting is the fact that GOWANS and his co-workers (see 15 for references) working on rats were able to demonstrate in a most elegant manner the immunologic role of the small lymphocyte. On the other hand a detailed examination of the quantitative course of haemopoiesis in guinea pigs led YOFFEY (see 16 for references) to believe that the lymphocytes may function as stem cells in myelopoiesis. GOODMAN (17) did not obtain bone marrow repopulation in X ray irradiated mice after transfusion of lymph node cell suspension, in direct opposition to the results obtained by one of us in rabbits (18) No explanation may be offered at this stage.

It is customary in experimental work to evaluate myelopoietic reactions on the basis of peripheral blood and bone marrow examinations. An accessory site of compensatory myelopoiesis—the spleen—is apt to be overlooked. Previous work on myelopoietic

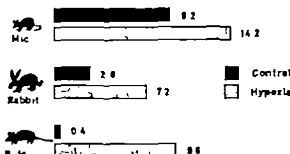



*Fig. 12.* Percentage of erythroblasts and granulocyte precursors in spleen imprint preparations in normal and hypoxic (6 days at 6000 m) animals. Myelopoietic reaction in the spleen in high altitude hypoxia.

reactions of the spleen in high altitude hypoxia (19) led us to believe that the role played by this organ may be quite important and varies according to species (cf. Fig. 12). In a similar experiment SAATHOF (20) obtained in guinea pigs comparable results to those in rabbits, as shown in Fig. 12. Later in the day the intensity of myelopoietic reaction in the spleen following the same relative blood loss in mice, rats and rabbits will be discussed (20).

A survey of literature, as well as personal experience, demonstrate that in rabbits (22) and guinea-pigs (23) heterotopic myelopoiesis accompanying induced osteogenesis is easily provoked. The same is true to a certain extent in dogs (24), but not in mice and rats (25). This may be related to the reactivity of primitive reticulum cells in MAXIMOW's sense, which seems to be concerned in these phenomena. It is too early to attempt a classification of laboratory animals according to the facility of inducing heterotopic myelopoiesis, nevertheless it seems to be well established that species differences exist in this respect.

Putting laboratory animals out of sight for a moment—it should be kept in mind that even mammals may have surprises in store. WORTH (26) found that in hibernating bats the bone marrow becomes aplastic and its function is taken over by the spleen and lymph nodes.

A simple comparison of the myeloid erythroid ratio (Fig. 13) and of the percentage of reticulum and lymphoid cells in the bone marrow of normal adult laboratory animals demonstrates marked differences. The reactivity of this organ also varies, if one is to

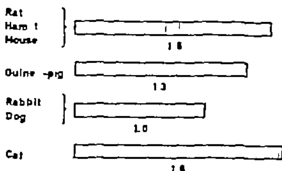



*Fig 13.* Myeloid/erythroid ratio in the bone marrow of laboratory animals according to (4)

studies on a few chosen species were performed using modern tracer methods for the evaluation of stem cell kinetics, erythrokinetics and the course of granulopoiesis. We did not find systematic comparative studies carried out by the same method with the aim to assess the differences or similarities in reactivity of the haemopoietic system. Here it should be stressed that such investigations offer one basic difficulty i.e. different sensibility of various species to the same experimental stimulus of the same intensity Perhaps the most demonstrative example is shown by the susceptibility to hypoxia. Rabbits and guinea-pigs die at a simulated altitude equivalent to 6000–7000 m, mice are slightly more resistent, rats may survive up to 15 min at 12 000 m (Fig 14)

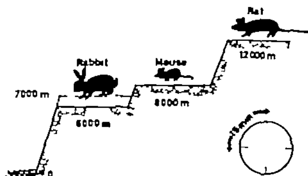



*Fig 14* Survival at high altitude.

Last but not least attention should be drawn to certain individual features of the haemopoietic system of the considered species. No explanation and possible bearing on experimental findings of the occurrence of large numbers of mast cells in rats, of the

presence of KURLOFF's bodies in guinea pigs and of the myelopoietic function of the spleen in normal adult mice are to be found in literature.

In selecting animals for a given experimental investigation and in evaluating the results several factors must be considered. Haemopoietic reactions may be obscured by displacement of erythrocytes (release from blood reservoirs to a different degree) and of granulocytes (release from the lungs 27 and displacements between the blood and the tissues, see 28 for references). Seasonal and diurnal variations exist not only in wild, but also in laboratory animals (4). The specific reactivity of the bone marrow and the varying ability to form extramedullary foci of myelopoiesis should be kept in mind and, incidentally, much more investigated. In the order of frequency of occurrence of such foci the spleen, the liver, the kidneys, the lungs and the lymph nodes should be named as the possible sites of extramedullary myelopoiesis.

The species-specific susceptibility to the factors operative in the construed experimental system must be carefully assessed, this being perhaps the most difficult problem. The evaluation of the immunological reactivity may be helpful here.

In certain instances the experimental worker is limited by a very restricted choice. Hereditary anomalies occur only in a few species. We left this problem out intentionally because it is fully covered in literature (see 29 for references), mainly by UNDRITZ's publications (see 30 for references). Immunology of laboratory animals was also omitted because of the work of DUJARRIC DE LA RIVIÈRE and EYQUEM (31).

We have to apologize that no conclusions have been formed, as the only possible conclusion seems to be—much more work must be done on strictly comparative aspects of the haematology of laboratory animals using modern methods for claryfying dynamic aspects of haemopoiesis.


*Summary*

Laboratory animals differ in physiology, morphology, absolute and relative counts of blood and blood-forming system cells. The reactivity of this system varies also, according to species. This may explain discrepancies in results, where the experimental system is formally the same, but the object (animal species) is different. Instances of such discrepancies, based on personal experience, are given and possible explanations advanced. In the authors' opinion much more attention should be given to species specifity when selecting the species on which the planned experiments will be performed.

More research work on strictly comparative aspects of haematology of laboratory animals is needed to interpret correctly many of the results hitherto obtained.


## *Zusammenfassung*

Laboratoriumstiere unterscheiden sich in Physiologie, Morphologie, absoluten und relativen Zahlen der Zellen des Blutes und der blutbildenden Organe. Ferner variiert die Reaktionsweise dieses Systems von einer Species zu anderen. Dadurch erklären sich Diskrepanzen der Resultate bei formal gleichem experimentellem System aber verschiedenem Objekt (Tierspecies). Es werden auf Grund eigener Erfahrungen Beispiele solcher Diskrepanzen und die möglichen Erklärungen dafür angeführt. Nach der Ansicht der Autoren sollte den Besonderheiten der Species wesentlich mehr Aufmerksamkeit geschenkt werden bei der Auswahl der Tierart, an der die geplanten Experimente ausgeführt werden sollen. Für die korrekte Interpretation vieler bisheriger Ergebnisse sind mehr Untersuchungen über genaue vergleichende Aspekte der Hämatologie von Laboratoriumstieren notwendig.



## *Résumé*

Les animaux de laboratoire diffèrent les uns des autres par leur physiologie, leur morphologie, le nombre absolu et relatif des cellules du sang et des cellules des systèmes hématopoïétiques. La réactivité de ces systèmes varient aussi selon les espèces. Ceci explique la différence des résultats dans les cas où le système expérimental est formalement le même mais l'objet (l'espèce animale) diffèrent. Des exemples de telles différences, reposant sur des expériences personnelles, sont présentés et les explications possibles en sont données. Selon l'opinion de l'auteur plus d'attention devrait être vouée aux propriétés spécifiques d'une espèce lors du choix de celle sur laquelle les expériences envisagées seront exécutées. Plus de travail de recherche concernant uniquement les aspects de l'hématologie des animaux de laboratoire est nécessaire afin de pouvoir interpréter correctement bien des résultats obtenus jusqu'à présent.


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# African Macroglobulinaemias

C Trincão J dos Santos Nascimento and
N Cordeiro Ferreira

Charmot and André have recently (1964) divided macroglobulinaemias into three groups

1 Macroglobulinaemias consecutive to infectious or parasitic diseases

2. Macroglobulinaemias of unknown aetiology with hyper gammaglobulinaemia and

3 Autonomous macroglobulinaemias or Waldenström's disease.

In a report on the macroglobulinaemias of the Africans the first two groups are solely to be considered and within them only the autochtonous diseases of Africa.

However before going into the subject some remarks concerning the peculiarities of the serum protein pattern in Africans seem pertinent.

In the last 20 years serum protein patterns of Africans have been studied regarding their total amount, the albumin globulin ratio and the several fractions isolated by electrophoresis and recently by immuno-electrophoresis. Results show clear cut differences between the so-called normal African and normal Europeans or Americans under the above-mentioned aspects (Symul, 1950 Van Oye and Charles, 1951 Holemans and Martin 1953 etc.) consisting in normal or decreased serum protein contents with a constant decrease of serum albumin and a constant increase of serum globulin.

Among globulin fractions $\gamma$ globulin is always greatly increased.

It has been discussed whether this particular protein pattern is due to racial factors. Already in 1950 Symul could state that newborn natives of the Belgian Congo had the same protein pattern as the Caucasian concluding that variations reported in adults were due to different conditions of life. The change of pattern occurs

between the 1st and the 2nd years (BERGOT and BASCOULERGUE). After examining 30 students from Gambia living in England SCHOFIELD (1957) stated that environment is able to transform the African serum protein pattern into the European though very slowly since such a change was not yet completed in a group of students living in England for more than 4 consecutive years.

In about 1500 American negroes, MILAM (1946) also observed a lower albumin globulin-ratio than in the white population. However these studies were made in North Carolina where according to the author the nutritional state of the population was that prevailing at the time in the region, the mean intake of proteins by adults being from 60 to 90 g daily. In the case of the students examined by SCHOFIELD (1957) this author thinks that the very slow change of their protein pattern is more probably attributable to partly reversible pathologic conditions, among which those caused by malaria seemed to fulfil an important role than to adaptation to environment differences such as alimentary proteins. VAN OYE and CHARLES (1957) are positive that really healthy Africans have similar serum protein patterns as the Caucasians. When interpreting data from the African population we must however bear in mind that they most probably regard the so-called normal subjects whose protein pattern shows the described abnormalities.

*Sleeping Sickness*

The only autochtonous macroglobulinaemia consecutive to a parasitic disease of Africa is that observed in sleeping sickness.

Earlier studies for the serum proteins in African trypanosomiasis have shown normal total values accompanied by a greater decrease of the albumin globulin ratio (SICÉ *et al.*, 1931. TRINCÃO and GOUVEIA, 1950 etc.) which according to that which is stated above is rather non-characteristic. Besides plasma fibrinogen is always increased (TRINCÃO *et al.* 1953).

In 1952 the first studies of the electrophoretic pattern of sleeping sickness sera were published. GANZIN *et al.* in an unstated number of cases examined by paper electrophoresis observed a considerable increase of $\gamma$ globulins in the patients within the first stage of the disease and a less considerable one in cases of the nervous stage. Conversely serum albumin was remarkably decreased. These statements regard the infection caused by *T. gambiense* and were con

firmed by other investigators (TRINCÃO *et al.* 1953 LE GAC *et al.*, 1954) and found to be similar in infections by *T rhodesiense*

TRINCÃO *et al.* (1953) in a survey of 25 sera from sleeping sickness cases submitted to electrophoresis in the Amtweiler apparatus noticed in every instance the presence of an abnormal wave occupying an intermediate position between $\beta$ and $\gamma$ globulins, i.e. the position correspondent to fibrinogen in plasma electrophoresis and on account of this called that wave $\varphi$ Extreme and mean values found were as under

| | % values | | | | | |
|---|---|---|---|---|---|---|
| | Albumin | $\alpha$ | $\alpha$ | $\beta$ | $\varphi$ | $\gamma$ |
| Maximum | 36 | 7 4 | 11,8 | 8,7 | 19,5 | 58,5 |
| Minimum | 18,3 | 1 1 | 1,3 | 4 1 | 3,2 | 42,5 |
| Mean | 28,4±5,4 | 4,6±1,5 | 4,9±4,6 | 5,7±1,3 | 8,8±6,7 | 47,8±4,7 |

The presence of this abnormal wave in serum electrophoretic diagrams of negro patients without sleeping sickness has been recorded by several authors LE GAC *et al.* noticed it in apparently healthy individuals as well as in cases of leprosy and trypanosomiasis, VIOLLIER and STAUB in cases of acute and chronic hepatitis and of liver cirrhosis, HOLMES in kwashiorkor It does not seem however to be as common a component of sera of apparently healthy negroes as could be deducted from LE GAC *et al* TRINCÃO *et al* (1953) who did not find it in 15 cases of ancylostomiasis studied by the Amtweiler apparatus, and from a search made in the archives of the department of Nutrition of the Institute of Tropical Medicine, Lisbon, their frequency on paper electrophoresis in apparently healthy Africans is the following

| | |
|---|---|
| Cape Verde | 7 in 107 sera (6,5 %) |
| Portuguese Guinea | 43 in 1273 sera (2,3 %) |
| Saint Thomas Island | 1 in 96 sera (1,04 ) |
| Angola | 21 in 609 sera (3,5 %) |

In 1950 DREYFUS *et al.* published a most interesting case of African trypanosomiasis in a whithe patient in whom Waldenström's macroglobulinaemia was diagnosed at first. As a matter of fact, as the authors point out, some of the symptoms of this disease such as adenopathies and splenomegaly occur in sleeping sickness, hemorrhages being the only important missing sign. Besides

the patterns of the bone marrow spleen and lymph gland biopsies are similar On ultracentrifugation the serum of their patient revealed 15 of macroglobulins with a sedimentation rate of 17 4 and 25 Svedberg units, immuno-electrophoresis demonstrating a considerable amount of $\beta$2M globulin The correct diagnosis was made 4 months later on account of the finding of the parasites in the cerebro-spinal fluid and in the bone marrow

Several other investigators performed immuno-electrophoresis of the sera of sleeping-sickness patients on a large scale.

MATTERN *et al* (1961) similarly studied 40 patients 8 of the first and 32 of the second stage of the disease finding a considerable increase of the $\gamma$1M ($\beta$2M) fraction without alteration of the $\gamma$1A ($\beta$2A) fraction. A similar study was carried out by NICOLI *et al.* (1961) in 65 more patients, 16 of the haemo-lymphatic and 49 of the nervous stages of the disease, with identical results. In some cases the line of precipitation of the $\beta$2M globulin looked doubled in others straight. Simultaneously the $\beta$2A precipitation line was thinner and even disappeared

Specific treatment brought about the normalization of the electrophoretic pattern especially among the patients of the haemo-lymphatic stage sometimes in 15 days, generally in 2–3 months (BENTZ and MACARIO 1963) Patients in the nervous stage did not recover so rapidly normal patterns being seen only 8–18 months after the beginning of the treatment. Persistance of the abnormal pattern after treatment indicates either that this has not been successful and that a clinical relapse is to be expected or that a reinfection took place. In both cases chemotherapy will induce the serological cure of the patient (BENTZ and MACARIO 1963) Contrarily to what could be predicted the higher values of $\beta$2M globulin are the quicker they are normalized by treatment.

In cases of the nervous stage MATTERN (1962) and MATTERN *et al* (1964) found macroglobulins in cerebro-spinal fluid too This search has revealed traces of $\beta$2M globulin in 6 out of 60 patients in the haemo-lymphatic stage and higher concentration of this protein in all the 174 cases of the nervous stage. Among cerebro-spinal fluids of 355 miscellaneous cases, traces of $\beta$2M globulin were only found in 4 patients with nervous syphilis. This is an important feature of the disease and one can only stress its diagnostic value in cases in which the aetiological agent cannot be found even after resorting to the most refined laboratory techniques.

firmed by other investigators (TRINCÃO *et al.*, 1953 LE GAC *et al.*, 1954) and found to be similar in infections by *T rhodesiense*

TRINCÃO *et al* (1953) in a survey of 25 sera from sleeping sickness cases submitted to electrophoresis in the Antweiler apparatus noticed in every instance the presence of an abnormal wave occupying an intermediate position between $\beta$ and $\gamma$ globulins, i.e. the position correspondent to fibrinogen in plasma electrophoresis, and on account of this called that wave $\varphi$ Extreme and mean values found were as under

| | % values | | | | | |
|---|---|---|---|---|---|---|
| | Albumin | $\alpha$ | $\alpha$ | $\beta$ | $\varphi$ | $\gamma$ |
| Maximum | 56 | 7 4 | 11,8 | 8,7 | 19,5 | 58,5 |
| Minimum | 18,5 | 1 1 | 1,5 | 4 1 | 3,2 | 42,5 |
| Mean | 28,4±5,4 | 4,6±1,5 | 4,9±[illegible],6 | 5,7±1,3 | 8,8±6,7 | 47,8±4 7 |

The presence of this abnormal wave in serum electrophoretic diagrams of negro patients without sleeping sickness has been recorded by several authors LE GAC *et al.* noticed it in apparently healthy individuals as well as in cases of leprosy and trypanosomiasis, VIOLLIER and STAUB in cases of acute and chronic hepatitis and of liver cirrhosis, HOLMES in kwashiorkor It does not seem however to be as common a component of sera of apparently healthy negroes as could be deducted from LE GAC *et al* TRINCÃO *et al* (1953) who did not find it in 15 cases of ancylostomiasis studied by the Antweiler apparatus, and from a search made in the archives of the department of Nutrition of the Institute of Tropical Medicine, Lisbon, their frequency on paper electrophoresis in apparently healthy Africans is the following

| | |
|---|---|
| Cape Verde | 7 in 107 sera (6,5 %) |
| Portuguese Guinea | 43 in 1273 sera (2,3 %) |
| Saint Thomas Island | 1 in 96 sera (1,04 %) |
| Angola | 21 in 609 sera (3,5 %) |

In 1950 DREYFUS *et al* published a most interesting case of African trypanosomiasis in a white patient in whom Waldenström's macroglobulinaemia was diagnosed at first. As a matter of fact, as the authors point out, some of the symptoms of this disease such as adenopathies and splenomegaly occur in sleeping sickness, hemorrhages being the only important missing sign. Besides

We studied the sera of 14 proved cases of sleeping sickness kindly made available by the Department of Tropical Pathology and Clinic of the Institute of Tropical Medicine of Lisbon and the Mission against sleeping sickness and other endemic diseases of Portuguese Guinea.

In every case the Sia test was positive demonstrating the euglobulinic nature of the paraproteins found.

Other methods of research were analytical ultracentrifugation * (Philpot's ultracentrifuge, circa 49 000 rotations at 20° lectures every 10 min) and immuno-electrophoresis (Scheidegger's micro-technique, polyvalent and anti β2M antisera from the Institute Pasteur of Paris).

Ultracentrifugation demonstrated two waves of macroglobulins, one corresponding to 10.8–15.8 Svedberg units and the other to macroglobulins with 19 Svedberg units or of bigger molecule. Amounts of the first fraction were variable, slightly predominating in the 7 cases of the haemo-lymphatic stage of the disease and never exceeding 15 % of the total proteins. In no case had this wave the configuration of a sharp peak as described in Waldenström's macroglobulinaemia. The second fraction varied between 2,8 and 12 % (mean 6,1 %) only in 1 patient being below 3 %.

Immuno-electrophoresis demonstrated in all but 1 case an intensification of the β2M line which sometimes had two segments or was forked in one of its extremities. With polyvalent antiserum the precipitation line correspondent to β2A globulin was in most of the cases very faint and never more intense that in normal sera (Fig. 1 and 2).

The significance of the increase of β2M globulin in the sera of sleeping-sickness patients remains to be fully explained. It may possibly depend on the stimulation of the activity of the plasmocytes which proliferate in this disease and is supposed to facilitate phagocytosis of trypanosomes by macrophages. Simultaneously information reaches one or more clones of immunologically competent cells inducing them to segregate the specific antibodies. Afterwards chemotherapy will bill bring the β2M globulin to normal levels without interfering with the production of specific antibodies (CHARMOT and ANDRÉ, 1964).

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* Examinations performed at the Laboratory of Veterinary Pathology.

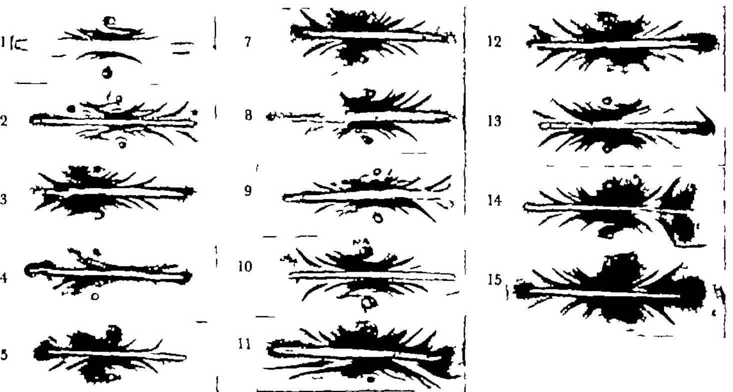



*Fig. 1* Serum immuno-electrophoresis 14 sleeping-sickness patients (polyvalent antiserum)

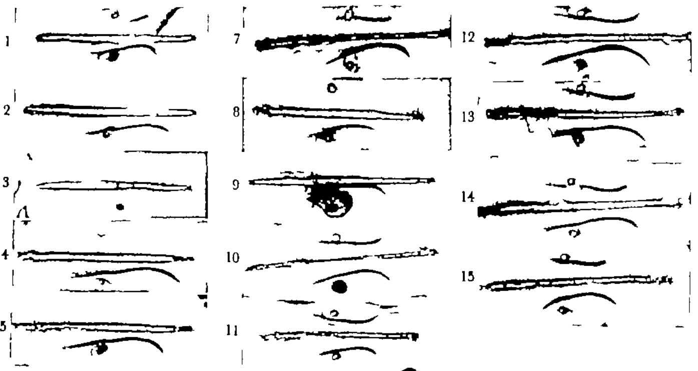



*Fig 2* Serum Immuno-electrophoresis in 14 sleeping-sickness patients (anti β2M-serum)

*Splenomegalies with Macroglobulinaemia*

Finally we must discuss the macroglobulinaemias of unknown aetiology with hypergammaglobulinaemia, which can be observed in the African population, independently of actual infective diseases.

In 1957 CHARMOT *et al* drew attention to these apparently idiopathic macroglobulinaemias from which 2 years later he had already observed some 10 cases. Most of them occurred in young subjects on poor health conditions with pale conjunctivae or slight subicterus, splenomegaly and a variable degree of hepatomegaly but without signs of liver cirrhosis. A similar case was described by FOUCHET in Madagascar (1960)

In 1962, ARON and VARGUES assembled and reported 26 cases of macroglobulinaemia with splenomegaly in Africans from Algeria, the French Congo and the Ivory Coast and tried to isolate a syndrome which they called macroglobulinaemia of the African type or CHARMOT's disease, suggesting that this nosological entity together with WALDENSTRÖM's macroglobulinaemia should be considered as primary macroglobulinaemias.

Both diseases differ in their course. In CHARMOT's syndrome symptoms are few asthenia, deterioration of the general condition of the patients which nevertheless does not prevent them from accomplishing their professional work, and splenomegaly without intrasplenic hypertension. In 17 of the reported cases spleens were considerably increased being palpated at the umbilical line or even below it and moderate hepatomegaly was observed in 15 cases. Oedema of the ankles could sometimes be noticed. In peripheral blood there was slight anaemia with lympho-monocytosis and frequently eosinophilia. In the bone marrow lympho-plasmocytosis could be detected in the absence of ROHR's lymphoid reticular cells. Biopsies of liver and spleen demonstrated non specific reticulo-endothelial hyperactivity Dysproteinaemia although similar to that of Waldenström's disease shows the following pecularities

1 On paper electrophoresis the spot of the globulins is wider in Charmot's syndrome.

2. On immuno-electrophoresis the double precipitation line of the $\beta$2M globulin typical of WALDENSTRÖM's disease is not seen in the African cases.

3 On ultracentrifugation macroglobulins in CHARMOT's syndrome do not exceed 10–20 of the total proteins and their aspect

in the diagram is that of an enlarged curve quite different from the narrow peak characteristic of WALDENSTRÖM's macroglobulinaemia.

Whilst Waldenström's macroglobulinaemia is to ARON and VAROUTA of a neoplastic nature in CHARMOT's syndrome, macroglobulinaemia is rather of the inflammatory type having a much slower course and is to be interpreted as a mere symptom of reaction to some inflammatory disease.

Two papers dealing with important numbers of patients on this syndrome were published in 1962–63.

In the first DEU *et al.* present a study of 28 cases. Splenomegaly was the only constant physical symptom in all the patients. Blood examinations revealed red cell counts between 2 100 000 and 4 600 000 per cm³ and anaemia when present was of the normochromic type. Furthermore leucopenia and neutropenia were frequent. The bone marrow revealed hypercellularity with a certain degree of erythroblastosis, moderate lymphocytosis and plasmocytes above the normal figures. No lymphoid reticular cells were observed. Liver biopsy disclosed an non-characteristic hyperplasia of the Kupffer cells and lymphoplasmocytic infiltrates. In 3 instances patterns of post-necrotic cirrhosis could be observed. The spleen was of the fibro-congestive type. Adenograms did not show important abnormalities.

The percentages of macroglobulins were the following

| Nr of cases | S 12,8 S 16,2 Extreme values | Mean | Greater than S 22 Extreme values | Mean |
|---|---|---|---|---|
| 18 | 9–30 | 14,6 | 1–4 | 1,3 |

On ultracentrifugation the peak of the macroglobulins was flat and had an enlarged base.

The authors conclude that Charmot's macroglobulinaemia is a particular feature of some of the splenomegalies that can be observed in Africa and for which an aetiology must be searched.

PAYET *et al.* (1965) studied the incidence of macroglobulinaemia and estimated immuno-electrophoretically its amount in 100 cases of miscellaneous liver and spleen diseases.

Out of 32 cases of primitive cancer of the liver only 7 had hypermacroglobulinaemia and among these 4 had their macroglobulins increased twice, 2 four times and 1 sixteen times.

Among 18 cases of liver cirrhosis 12 were macroglobulinaemic, 4 with twice the normal amounts of macroglobulinaemia, 5 four times, 1 eight times and 2 sixteen times that value. The increase in macroglobulins could not be correlated with the severity of the disease. Among 9 patients with splenomegaly 5 had a significative increase in macroglobulins. Conversely among 9 cases without splenomegaly 7 had abnormal immuno-electrophoretical diagrams though the increase in $\beta 2M$ globulin was moderate in 6 instances.

On the other hand only 22 from 50 cases of the so-called African splenomegaly had hypermacroglobulinaemia in 7 cases twice the normal value, in 5 four times and in 10 eight times.

Neither the degree of splenomegaly nor the haematological data, or the results of liver biopsy or those of splenic portography or manometry explain the pattern of the immuno-electrophoretic analysis of the sera.

Such findings stress the lack of homogeneity within this group of patients and on account of it the authors believe that among them macroglobulinaemia is a serologic feature which cannot be predicted on the grounds of clinical or haematological findings. Moreover splenectomy does not induce the cure of the serological abnormality

Considering these tropical splenomegalies together with the wide group of diseases in which macroglobulinaemias have been found CHARMOT and ANDRÉ (1964) point out that these are generally considered as self entertained diseases with auto-immunization as their common denominator In such cases macroglobulins would act by transporting antigen antibody complexes and facilitating their phagocytosis by the macrophages of the reticulo-endothelial system. In the particular case of tropical splenomegalies macroglobulinaemia does not seem to correspond to the synthesis of any immune antibody This would explain the lack of correlation between their titre with the clinical symptoms together with their relationship with lymphocytic and reticulo-endothelial reactions.

### *Summary*

A review of the literature concerning African macroglobulinaemias allows their division into two groups one homogeneous connected with sleeping sickness and another perhaps heterogeneous related splenomegalies of unknown aetiology

We have assessed the importance of this symptom in 15 cases of sleeping sickness by studying their sera by ultracentrifugation and immuno-electrophoresis. Ultracentrifugation *demonstrated two waves of macroglobulins, one corresponding to 10,8–*

15,8 Svedberg units and the other to macroglobulins of 19 Svedberg units or of bigger molecule. Amounts of the first fraction were variable never exceeding 15 % of the total proteins and their wave not having the configuration of sharp peak. The second fraction varied between 2,8 and 12 % (mean 6,1 %) Immuno-electrophoresis revealed in all but 1 case an intensification of the β2M line sometimes with two segments or forked in one extremity

## Zusammenfassung

Eine Durchsicht der Literatur über die afrikanischen Makroglobulinämien erlaubt eine Einteilung derselben in zwei Gruppen eine homogene mit Beziehungen zur Schlafkrankheit und eine wahrscheinlich heterogene in Verbindung mit Splenomegalien unbekannter Aetiologie.

Wir versuchten, die Bedeutung dieser Symptome zu beurteilen bei 15 Fällen von Schlafkrankheit durch Untersuchung ihrer Seren mit Hilfe der Ultrazentrifuge und der Immunelektrophorese. Die Ultrazentrifugierung ergab zwei Wellen von Makroglobulinen, wovon die eine 10,8 – 15,8 Svedberg-Einheiten entsprach und die andere Makroglobulinen mit 19 Svedbergeinheiten oder mit größerem Molekül. Die Mengen der ersten Fraktion wechselten und überstiegen nie 15 % der gesamten Proteine, und ihre Welle bildete keine scharfe Spitze. Die zweite Fraktion variierte zwischen 2,8 und 12 % (Mittel 6,1 %) Die Immunelektrophorese ergab in allen Fällen mit einer Ausnahme eine Verstärkung der β2M-Linie die bisweilen in zwei Segmente geteilt oder am einen Ende aufgespalten war

## Résumé

La révision de la littérature concernant les macroglobulinémies africaines permet leur division en deux groupes un homogène en rapport avec la maladie du sommeil, l'autre probablement hétérogène ayant trait à des splénomégalies d'étiologie inconnue.

Nous avons évalué l'importance de ce symptôme chez 15 cas de maladie du sommeil en étudiant leur sérum par ultracentrifugation et par immuno-électrophorèse. L ultracentrifugation démontré deux ondes de macroglobulines l'une correspondante à 10,8–15,8 unités de Svedberg et l'autre à des macroglobulines avec 19 ou plus unités de Svedberg Les pourcentages de la première fraction étaient variables, jamais excédant 15 % des protéines totales leur onde n ayant jamais l'aspect d'un pic aigu. La seconde fraction varié entre 2,8 et 12 % (en moyenne 6,7 %) L'immuno-électrophorèse décelé dans tous les cas 1 exceptée une intensification de la ligne de précipitation des globulines β2M parfois divisée en deux segments ou bifide à l'une de ses extrémités.

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# Die Atransferrinämien

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Unter Atransferrinämie verstehen wir eine extreme Verminderung von Transferrin im zirkulierenden Plasma. Es handelt sich also nicht – wie der Name sagt – um ein vollständiges Fehlen. Das ist bisher noch nie beobachtet worden. Eine sehr kleine Menge von Transferrin ist auch in extremen Fällen immer noch nachweisbar. Aber durch die enorme Verminderung des Transferrins werden Störungen im Eisenstoffwechsel sichtbar. In diesen Fällen können wir von *Atransferrinämie* sprechen. Wenn es sich nur um eine Verminderung von Transferrin handelt, ohne daß sichtbare Folgen im Eisenstoffwechsel auftreten, so bezeichnen wir diese Zustände mit *Hypotransferrinämie*.

In diesem Referat sollen jedoch nur diejenigen Fälle mit extremer Verminderung des Transferrins, also die Atransferrinämien, besprochen werden.

Grundsätzlich kann eine Verminderung des Transferrins entweder durch eine verminderte Bildung oder durch einen gesteigerten Abbau bzw. durch einen Verlust nach außen entstehen. Darüber hinaus hat MATHYUA noch auf eine Transferrinverminderung durch Abwanderung ins entzündete Gewebe aufmerksam gemacht. Alle diese Möglichkeiten sind bekannt. Sie sind in der folgenden Tabelle I dargestellt.

Weiterhin können wir die Atransferrinämien in kongenitale durch einen Error of metabolism bedingte und in erworbene Formen einteilen. Von besonderer Bedeutung ist die Kenntnis der durch den Wegfall des Transferrins hervorgerufenen Störungen im Eisenstoffwechsel, auf die wir jetzt näher eingehen wollen.

Ihr Studium ist am reinsten dann möglich, wenn die einzige Störung im Organismus nur in dem Wegfall des Transferrins besteht. Dieses Experimentum naturae liefert uns die kongenitale

*Tabelle I*

| | |
|---|---|
| Verminderte Synthese des Transferrins in der Leber | Krankheit<br>1. Kongenitale Atransferrinämie (HEILMEYER und Mitarb.)<br>2. Hypotransferrinämie bei Infekt oder bei Lebererkrankung<br>3. bei allgemeiner Hypoproteinämie. |
| Gesteigerter Abbau des Transferrins | beim Infekt, bei malignen Tumoren und Kollagenkrankheiten, |
| Abwanderung ins entzündete Gewebe | bei Entzündungsherden (so bei Tuberkulose) |
| Verlust nach außen | |
| a) durch die Nieren | nephrotisches Syndrom |
| b) durch den Darm | exsudative Enteropathie |

Atransferrinämie, von der wir bisher nur einen einzigen Fall kennen, den HEILMEYER – gemeinsam mit KELLER, VIVELL, KEIDERLING, BETKE, WÖHLER und SCHULTZE – veröffentlicht haben.

Es handelt sich um ein 7jähriges Mädchen, das mit normalem Gewicht und normaler Größe geboren wurde. Bereits im Alter von 3½ Monaten wurde eine starke *hypochrome Anämie* festgestellt und mit verschiedenen Eisenpräparaten sowie mit Bluttransfusionen behandelt. Die Anämie blieb jedoch völlig therapierefraktär gegenüber allen antianämischen Mitteln. Im weiteren Verlaufe zeigte sich eine Verzögerung des Wachstums und der gesamten Entwicklung. Es kam 17 mal in ein Krankenhaus, ohne daß sich die Ursache der Anämie aufklären ließ. Auch in der Universitäts-Kinderklinik, wohin das Kind schließlich eingewiesen wurde, gelang beim ersten Aufenthalt keine Klärung der Anämie. Erst als Blut zur *Immunelektrophorese* nach der Entlassung des Kindes eingesandt wurde, fiel das Fehlen des Transferrins auf.

Das Kind zeigte bei der Klinikaufnahme eine Größe von 106 cm statt normal 121,2 cm. Das Körpergewicht betrug 15,6 kg statt 22,6 kg. Auch die Zahnentwicklung war zurückgeblieben. *Leber* und *Milz* waren deutlich vergrößert und tastbar. Das *Leberpunktat* zeigte eine exzessive Siderose mit vermehrter Fibrose. Die Leberteste waren schwer pathologisch (Thymol ++++) Weltmann-Band 10 Röhrchen, Takata 60 mg SGOT 106 SGPT 60, SLAP 578

Das *Blutbild* war wie folgt: Hb 9,1 g%, Ery 4,1 Mill., $Hb_E$ 22. Im Blutausstrich stark hypochrome Erythrozyten mit vielen Target

zellen. Normale Leukozyten und Thrombozytenwerte. Im Sternalpunktat starke Vermehrung der Erythropoese mit zahlreichen basophilen polychromatischen Erythroblasten, wie bei Eisenmangelanämie. Im selben Sinne spricht die starke Verminderung der *Sideroblasten* (13 % sehr feinkörnige) gegenüber 30–50 % normal. Die *Retikulumzellen* waren völlig eisenfrei. Kein pathologisches Hämoglobin.

*Serum Eisen* zwischen 9 bis 14 $\mu$g/100 ccm Serum. *Serum Kupfer* 155 $\mu$g Totale Eisenbindungskapazität 33 $\mu$g

*Ferrokinetik* T/2 = 5 min (normal 70–140 min) Plasmaeisen Turn over 1 63 mg/kg/24 h (normal 0,36–0 52 mg/kg/24 h) also sehr stark erhöht. Nichthämoglobin Eisen Turn over 21 6 mg/24 h (normal 6,2 mg/24 h) Erythrozyten Einbaurate 10 % in 8 Tagen Oberflächenmessung nach intravenöser Gabe von Fe 59 Sehr hoher Eiseneinbau in die Leber mäßiger Einbau in die Milz, sehr geringer Einbau in das Knochenmark (nach Bebrütung mit dem körpereigenen Plasma der Patientin durchgeführt) Die Messung der Eisenresorption ergab eine Resorptionsgröße von 30 % Erythrozyten-Lebensdauer mit Chrom 51 T/2 = 24 Tage (normal 30) fast normal. Die Eiweißwerte Das Elektrophoresediagramm zeigt eine Verminderung der Beta Zacke nach Versetzen des Serums mit Fe 59 sieht man eine Radioaktivität über einer spitzen Zacke vor der Gamma Zacke (x Zacke) welche wahrscheinlich einen Antigen Antikörper Komplex mit Transferrin darstellt infolge der zahlreichen Transfusionen mit Plasma und Vollblut.

Die Immunelektrophorese wurde bereits eingangs gezeigt. Eine Immunelektrophorese mit reinem Antitransferrinserum zeigte keine Spur von Transferrinpräzipitation im kindlichen Serum. Eine quantitative immunologische Bestimmung des Transferrins, welche von Dr Schultze in den Behring Werken, Marburg durchgeführt wurde, ergab 0,054 % Transferrin, was etwa 1/100 der Norm entspricht.

Sowohl *beim Vater* wie *bei der Mutter* fand sich ein Transferringehalt, der etwa auf die Hälfte der Norm herabgesetzt war Daraus ist zu schließen, daß es sich bei der kongenitalen Atransferrinämie um eine autosomale rezessive Vererbung handelt. Das Krankheitsbild tritt nur bei Homozygotie in voller Gestalt in Erscheinung was auch die außerordentliche Seltenheit dieser Krankheit erklärt. Im weiteren Verlauf wurde durch Plasmainfusionen eine leichte Besserung des Gesamtzustandes und des Blutbildes erzielt. Nach der

Entlassung aus der Klinik starb das Kind jedoch auf der Heimreise unter den Zeichen eines plötzlichen Herzversagens.

Die Autopsie zeigte in allen untersuchten Organen eine außerordentliche Eisenspeicherung. Am stärksten war die Eisenanhäufung in der Leber und im Pankreas. Die enorme Eisenspeicherung der Leber hat zu einer kleinknotigen Lebercirrhose geführt, die auch klinisch nachweisbar war. In der Niere fand sich eine starke Eisenablagerung in den Epithelien der Harnkanälchen. Besonders die Schaltstücke und die Zwischenstücke zeigten reichlich Eiseneinlagerungen. Auch in der Lunge war vermehrt Eisen abgelagert, besonders in den Bronchialepithelien und den Schleimdrüsen. Im Herzmuskel fand sich eine schwerste herdförmige Hämosiderose mit Eisen in den Herzmuskelfasern. Dabei ausgedehnte, teilweise fleckförmige, teilweise diffuse fein- bis grobstreifige Verschwielung des Herzmuskels. Offenbar hat die Eiseneinlagerung diese schwere Schädigung der Herzmuskelfasern bewirkt, welche auch klinisch in Form einer starken Dilatation des Herzens (Rö. Bild) und im Elektrokardiogramm nachweisbar war. Letzten Endes ist das Kind an der Eisenüberladung der Herzmuskelfasern und den dadurch bedingten Nekrosen gestorben.

Im Gegensatz zu dieser schweren Eisenüberladung aller untersuchten Organe zeigt eine Eisenfärbung des Knochenmarks ein nahezu vollkommenes Fehlen des Eisens. Dies rührt offenbar daher, daß das eisenhungrige erythropoetische Gewebe einen starken Eisensog ausübt und auch die Retikulumzellen des Knochenmarks zur Entleerung ihres Eisens zwingt.

Dieser reine Fall einer kongenitalen Atransferrinämie vermittelt uns ganz im Sinne eines Experimentum naturae ein klares Bild einer Eisentransportstörung. Wenn das eisenbindende Protein fehlt, wird das Eisen nicht genügend im Plasma fixiert. Es verläßt allzu rasch die Blutbahn. Das Eisen gelangt in alle Organe und häuft sich dort an. Während normalerweise etwa 80% des zirkulierenden Eisens ins Knochenmark gelangen, wo es mit Hilfe spezifischer Rezeptoren von den Erythroblasten und Retikulumzellen aufgenommen wird, gelangen hier nur 8–10% des zirkulierenden Eisens an das Knochenmark. Das Knochenmark ist an Eisen verarmt, und es entwickelt sich das paradoxe Bild einer Eisenmangelanämie, während alle übrigen Organe voll mit Eisen beladen sind. In der Leber kommt es durch die Eisenüberladung zum Untergang von Parenchymzellen und zur Entwicklung von Bindegewebe. Es ent-

steht das Bild einer siderotischen Leberzirrhose. Besonders gefährlich ist die Eiseneinlagerung in dem Herzmuskel, welcher dadurch schwer geschädigt wird und plötzlich zum Versagen kommt. So sterben die an einer Eisenmangelanämie leidenden Patienten letzten Endes an der Eisenüberladung der übrigen Organe, besonders des Herzens. Zweifellos wurden diese Vorgänge durch die verschiedenen Bluttransfusionen und Eisengaben die in Verkennung der Situation dem Kinde zahlreich gegeben worden sind beschleunigt. Aber letzten Endes wäre auch ohne diese äußere Eisenzufuhr allein infolge der Transportstörung das Bild zustandegekommen.

Diagnostisch ist die Erkrankung leicht festzustellen wenn man nur an die Möglichkeit denkt. Man sollte deshalb bei jeder eisen refraktären Anämie, wie hypochromen Anämie mit niederem Serumeisen durch Leberpunktion nach der Hämosiderose in der Leber sehen. *Noch einfacher und sicherer ist die Bestimmung der* totalen Eisenbindungskapazität, welche bei der Atransferrinämie außerordentlich klein ist und sich etwa mit dem stark verminderten Serum-Eisen deckt, während beim echten Eisenmangel die totale Eisenbindungskapazität sogar über die Norm erhöht ist.

Therapeutisch sollten wir mit Plasmatransfusionen oder Transfusionen von reinem Transferrin die Transportstörung zu beseitigen suchen. Eisengaben sollten jeweils nur zusammen mit Plasmatransfusionen gegeben werden. Ist es bereits zu einer starken Eisenanhäufung gekommen, so sollte mit Desferrioxamin eine Enteisenung der Organe vorgenommen werden.

### *Erworbene Atransferrinämien*

Während die Hypotransferrinämien bei den verschiedensten Erkrankungen häufig vorkommen (siehe die eingangs gezeigte Tabelle) sind die schwersten Formen, die man als Atransferrinämie bezeichnet, sehr selten. Der erste Fall dieser Art wurde von Riegel und Thomas in USA beschrieben. Es handelte sich um eine 85jährige Patientin mit hochgradiger hypochromer Anämie von 3,1 g Hb. und 1,04 Mill. Ery die an einer Pyelozystitis und Pyonephrose mit Abszessen und einem Ureterstein litt. Der Tod trat 3 Jahre nach Entwicklung des schweren Krankheitsbildes ein Die Autopsie ergab eine hochgradige Siderose der Milz, der abdominellen Lymphknoten und der Leber In der Leber war eine lobuläre Fibrose nachweisbar Das Knochenmark war zellreich. Die Papier

elektrophorese der Plasmaeiweißkörper ergab eine extrem vermin derte Beta-Globulinfraktion. Der Eisengehalt des Serums betrug 75 µg wahrscheinlich nach Transfusion bestimmt. Die latente Eisenbindungskapazität war Null. Auf Eisentherapie war die Pa tientin refraktär. Insgesamt hat sie 640 Bluttransfusionen erhalten. Die Autoren glauben, daß das Fehlen des Transferrins durch eine darniederliegende Proteinsynthese infolge der schweren infektiösen Erkrankung bei dem hohen Alter bedingt war. Die schwere Anämie wurde von den Autoren durch Wegfall der Eisentransportfunktion des Beta-Globulins (also des Transferrins) erklärt.

Ein zweiter Fall mit stärkster Verminderung des Transferrins wurde von Hitzig, Schmidt, Betke und Rothschild mitgeteilt. Es handelte sich um ein zwei Jahre und 9 Monate altes Kind, bei dem eine akute Erythrämie Di Guglielmo mit Wucherung der Para erythroblasten im Knochenmark, in Milz und Leber vorlag. Eine Organsiderose bestand hier nicht. Die stark hypochrome Anämie war mäßig (9,6 g% Hb.). Das Serum-Eisen lag bei 27 µg%. Die latente Eisenbindungskapazität war Null. Immunchemisch wurde eine sehr geringe Menge Transferrin von etwa 1/30 der Norm nachgewiesen. Der Transferrinmangel wird mit der Erythrämie (Bildungsstörung) in Zusammenhang gebracht.

Eingehend untersucht ist in meiner Freiburger Klinik ein neuerdings beobachteter Fall von extremem *Transferrinmangel bei einer Nephrose*. Es handelt sich um eine 14 Jahre alte Patientin, welche im Gefolge einer Glomerulonephritis ein nephrotisches Syn drom mit einer Proteinurie von 20‰ bekam, was etwa 20–30 g Eiweißverlust täglich entsprach. Gleichzeitig entwickelte sich eine Anämie von 9,5 g% Hb und 2,6 Mill. Ery. Die Erythrozyten waren teilweise stark hypochrom. Das Elektrophoresediagramm zeigte eine extreme Verminderung des Albumins, sowie der Beta- und Gamma globuline. Nur Alpha 2 war relativ stark vermehrt. Das Elektrophoresediagramm des Harns zeigt das umgekehrte Verhalten, starke Albumin- und Gammaglobulinausscheidung, dagegen eine sehr geringe Ausscheidung von Alpha 2.

Immunelektrophoretisch waren im Serum Albumin stark ver mindert, ebenso das Transferrin. Im Harn konnte dagegen Transferrin deutlich nachgewiesen werden. Die Bestimmung des Transferrinumsatzes mit $^{131}I$ markiertem Transferrin ergab eine stark beschleunigte Plasmaabwanderung mit einer Halbwertszeit T/2 = 2,8 Tage statt normal 8,4 Tage. Diese beschleunigte Abwanderung

des Transferrins aus dem Plasma war in erster Linie durch den Verlust durch die Nieren bedingt. Der tägliche Verlust durch den Harn wurde zu 310 mg Transferrin täglich berechnet. Der Transferrin-Katabolismus war gleichzeitig gegenüber der Norm gesteigert (291 mg/Tag) was einer Halbwertszeit von 6 Tagen statt 8–10 Tagen entspricht. Der Verlust durch den Harn steht aber an erster Stelle. Wir haben in diesem Falle die verschiedenen Eiweißkörper im Harn und im Serum bestimmt. Wie die Tabelle II zeigt, werden alle Eiweißkörper mit dem Molekulargewicht bis 160 000 im Harn ausgeschieden, während die makromolekularen Eiweißkörper mit einem Molekulargewicht von 800 000 bis 3 000 000 die Glomerulusmembranen nicht passieren können. Das Transferrin mit einem Molekulargewicht von 88 000 das nur wenig größer ist als das Molekulargewicht des Albumins, passiert die Glomerulusmembran sehr leicht.

*Tabelle II*

Ausscheidung einzelner Proteinfraktionen im Urin

| Ausscheidung | | Fehlende Ausscheidung | |
|---|---|---|---|
| Proteinfraktion | Mol.-gew | Proteinfraktion | Mol.-gew |
| Albumin | 69 000 | Haptoglobin | polymorph |
| $\alpha_1$-saures Glycoproteid | 44 000 | $\alpha_2$-Makroglobulin | 800 000 |
| | | $\alpha_1$- und $\beta$-Lipoproteide | ca. 1 000 000 – 3 000 000 |
| Coeruloplasmin | 150 000 | $\gamma_{1M}$-Globulin | ca. 1 000 000 |
| Transferrin | 88 000 | | |
| Hämopexin | 80 000 | | |
| $\gamma_G$-Globulin | 160 000 | | |

Dieser dauernde Verlust durch die Nieren und der gesteigerte Katabolismus des Transferrins können durch eine gesteigerte Synthese nicht mehr ausgeglichen werden So kam es zur extremen Verminderung des Transferrins im Serum.

Die Eisenstoffwechseldaten waren folgende

Serum Eisen 21 $\mu$g

Latente Eisenbindungskapazität 3 $\mu$g

Totale Eisenbindungskapazitat *24 $\mu$g!*

Fe 59 – Abwanderung aus dem Plasma T/2 = 21 min (normal 80–140 min)

Eiseneinbau in die Erythrozyten am 7 Tag 52 % (normal 70–90 %)

Eisenresorption aus dem Darm 87 % (normal 25–50 %)
Sideroblasten im Knochenmark 12 % (stark vermindert)
Retikulumzellen des Knochenmarks Starke Eisenspeicherung +++

Die Strahlenmessung von außen nach Fe 59 zeigte einen primären Anstieg über dem Kreuzbein in den ersten Stunden mit raschem Abfall. Ein geringerer Anstieg in der Leber ein stärkerer über der Milz. Die Kurven können so gedeutet werden, daß zwar dem Knochenmark noch relativ größere Mengen Eisen zuströmen daß aber das Eisen nur zum Teil der Erythropoese dient. Ein großer Teil des Eisens gelangt in die Retikulumzellen des Knochenmarks, ebenso in die Retikulumzellen von Milz und Leber Die histologische Untersuchung hat tatsächlich eine deutliche Eisenspeicherung in diesen Retikulumzellen ergeben, die jedoch im Vergleich zu dem Falle von kongenitaler Atransferrinämie sehr viel geringer ist. Außerdem findet sich keine Eisenspeicherung in den Parenchymzellen. Die Ursache liegt in den starken Eisenverlusten durch den Harn. Gleichzeitig dürfte eine Entzündungssituation mit gesteigerter Affinität der Knochenmarksretikulumzellen zum Eisen bei diesem Fall eine Rolle spielen, ein Vorgang den jede Entzündungsreaktion begleitet (beim Infekt, bei malignen Tumoren und Kollagenkrankheiten) So unterscheidet sich das Bild der erworbenen Atransferrinämie in wesentlichen Zügen von der kongenitalen Atransferrinämie.

*Bei der kongenitalen Form* finden wir eine totale Verarmung des Knochenmarks an Eisen, sowohl der Retikulumzellen, wie der Erythroblasten. Dagegen sind die übrigen Organe, RES-Zellen und Parenchymzellen außerordentlich stark mit Eisen überladen.

Bei der erworbenen Form (durch Nephrose) ist im Knochenmark die Eisenspeicherung in den Retikulumzellen vorhanden dagegen ist das Eisen der Erythroblasten sehr vermindert (niedere Sideroblastenzahlen) Die übrigen Organe sind in den Parenchymzellen völlig eisenfrei. Geringe Eisenspeicherung im RES *Die Gefahr eines Todes durch Eisenüberladung besteht also bei der erworbenen Form durch Nephrose nicht.* In beiden Fällen ist die Eisenresorption unbehindert, im Falle der Nephrose sogar erheblich gesteigert. Das ist ein Beweis dafür daß das Transferrin keine wesentliche Rolle für die Aufnahme des Eisens aus dem Darm in das Blut spielt. Bei den erworbenen Formen hängt die Behandlung natürlich vom Grundleiden ab Dieses gilt es zu bessern. Eine Enteisenung braucht bei

den nephrotischen Formen nicht durchgeführt zu werden. Hier können Bluttransfusionen, Plasmatransfusionen sowie auch Eisengaben ohne Bedenken durchgeführt werden.

Das Krankheitsbild der Atransferrinämie ist zwar selten. Aber die Erforschung der pathophysiologischen Zusammenhänge ist äußerst interessant, weil hier eine Experimentum naturae vorliegt, wie es schöner im Laboratorium nicht erzeugt werden kann. So haben diese Fälle von Atransferrinämie unser Wissen über den Eisenstoffwechsel, besonders über die Transportfunktion des Transferrins und über die Bedeutung bei der Eisenresorption aus dem Darm wesentlich bereichert.


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Es wird über die Transferrinmangelzustände im Blutplasma berichtet. Diese teilen sich in kongenitale und erworbene Formen.

Bei der kongenitalen Atransferrinämie kommt es zu schweren Störungen des Eisentransports. Das Eisen lagert sich in allen Organen ab, während das Knochenmark zu wenig Eisen für die Hämoglobinsynthese erhält. Dadurch kommt es zu einer therapierefraktären hypochromen Anämie mit extrem niedrigen Serum-Eisen-Werten.

Die erworbenen Formen beruhen entweder auf Transferrinverlusten durch den *Darm (exsudative Enteropathie) oder durch die Niere (Nephrosen)* oder auf verminderter Transferrinsynthese (b. Hypoproteinämien, Infekten u. a.). Auch ein gesteigerter Abbau von Transferrin und eine erhöhte Abwanderung in das entzündete Gewebe können zu Transferrinmangelzuständen führen.

## *Summary*

The different forms of transferrin deficiency in the plasma are discussed. There are congenital and acquired types.

Congenital atransferrinaemia gives rise to severe disturbances in iron transport. Iron is deposited in all organs, while the bone marrow contains too little for haemoglobin synthesis. The consequence is refractory irondeficient anaemia with extremely low serum iron levels.

Acquired forms are due to either enteric (exudative enteropathy) or renal (nephropathy) transferrin loss, or to impaired synthesis of transferrin (hypoproteinaemia, infection etc.). Increased breakdown of transferrin or migration to inflammatory tissues can also lead to deficiency.

## *Résumé*

*Les états déficitaires en transferrine font l'objet de ce rapport. Ils sont classés en* formes congénitales et acquises.

Dans les transferrinémies congénitales, le transport du fer est gravement perturbé. Le fer se dépose dans tous les organes, pendant que la moelle osseuse reçoit trop peu de fer pour la synthèse de l'hémoglobine. Ainsi s'établit une anémie hypochrome avec des taux de fer sérique très bas, anémie refractaire à la thérapeutique.

Les formes acquises sont dues à des pertes de transferrine soit par les intestins (entéropathies exsudatives) soit par les reins (néphroses) ou encore à une réduction de la synthèse de la transferrine (dans les hypoprotéinémies, les infections etc.) Une dégradation accrue de la transferrine et une déperdition augmentée dans les tissus enflammés peuvent mener à des états déficitaires en transferrine.

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### 3 Lebenszyklus der Lymphozyten

Die Markierung von Lymphozyten *in vivo* mit Hilfe von radioaktiven Aminosäuren hat in den letzten Jahren überraschende Erkenntnisse über ihren Lebenszyklus erbracht. Besonders aus den Arbeiten von GOWANS und Mitarb. geht hervor, daß ein gewisser Prozentsatz (ca. 80 %) der kleinen Lymphozyten außerordentlich langlebig ist und im Körper eine ständige Rezirkulation durchmacht. Aus Messungen an der Maus und Ratte wurde für diese Zellen eine Überlebensdauer von über 300 Tagen nachgewiesen, aus indirekten Befunden beim Menschen schließt man heute, daß sie mindestens 10 Jahre überleben können. In dieser Zeit sind die Lymphozyten in ständigem Kreislauf durch den Körper, der von den Produktionsstätten durch die efferenten Lymphbahnen in den Ductus thoracicus und von dort in den Blutstrom führt; aus den peripheren Blut-Gefäßkapillaren wandern die Lymphozyten durch aktive Bewegung in den interstitiellen Raum aus, in dem sie sich längere Zeit aufhalten können; man schätzt, daß etwa 99 % des gesamten Lymphozytenpools außerhalb der Lymphbahnen und Blutgefäße liegt. Durch Rückwanderung in die afferenten Lymphbahnen kann der Lymphozyt den Anschluß an den Kreislauf wieder erreichen.

Der Rest der kleinen Lymphozyten von etwa 20 % hat eine sehr kurze Lebensspanne von wenigen Stunden oder Tagen; über sein Schicksal ist weniger bekannt. Man weiß aber, daß alle kleinen Lymphozyten bei Übertragung in ein Versuchstier die lymphatischen Organe aufsuchen und sich dort ansiedeln oder in den beschriebenen Lymphozytenkreislauf eintreten.

Die großen Lymphozyten verhalten sich völlig anders: nach intravenöser Injektion sammeln sie sich innerhalb kurzer Zeit im lymphatischen System des Magendarmkanals, d. h. vor allem in den Peyer'schen Plaques an, in denen sie nur wenige Tage nachweisbar bleiben.

Die wichtigste Fehlerquelle der autoradiographischen Technik mit tritium markierten Aminosäuren liegt in der Reutilisation von radioaktivem Material. Die quantitative Bedeutung dieser Fehlermöglichkeit muß in Betracht gezogen und in jedem einzelnen Fall abgeschätzt werden. Im folgenden wird nicht weiter darauf eingegangen, es kann aber allgemein gesagt werden, daß in den zu zitierenden Untersuchungen dieser Umstand gebührend berück

Laufe der folgenden zwei Tage in großer Anzahl aus den großen pyroninophilen Zellen hervor und erreichen das Transplantat vom Blutweg aus. Sie grenzen es vom körpereigenen Gewebe ab und leiten seine Nekrose ein.

Konventionelle humorale Antikörper spielen bei der Transplantatabstoßung keine wesentliche Rolle. Entscheidend wichtig ist ein enger Kontakt der fremden Gewebe mit den Wirtslymphozyten, die auf diese Weise eine zytotoxische Wirkung ausüben, über deren genauen Mechanismus noch wenig bekannt ist; der zytophile Antikörper von BOYDEN und SORKIN könnte dabei eine wichtige Rolle spielen.

Die einmal sensibilisierten lymphoiden Zellen zirkulieren während vielen Monaten, woraus sich die beschleunigte anamnestische Reaktion erklärt.

*b) Die Reaktion von transplantierten lymphoiden Gewebe gegen den Wirtsorganismus (graft versus host reaction).* Dieser Vorgang wurde in vielen Varianten sehr genau studiert; er besteht in der immunologischen Reaktion der Donor-Lymphozyten gegen (ihnen fehlende) Antigene des Wirtes. Man hat sie deshalb auch als Homotransplantat-Reaktion mit vertauschten Rollen bezeichnet. Die auslösenden Donor-Lymphozyten können sowohl aus Lymphknoten oder Milz als auch aus peripherem Blut als auch aus dem Ductus thoracicus stammen. Durch die Fraktionierung der Zellsuspensionen wurde der stichhaltige Beweis dafür erbracht, daß die Aktivität des Inoculums seinem Gehalt an kleinen Lymphozyten parallel geht, aber keine Beziehung zur Anzahl der darin enthaltenen Monozyten aufweist.

Mit tritium-markierten Zellen konnte das Schicksal der injizierten Lymphozyten weitgehend geklärt werden: in den ersten 12–24 h sammeln sie sich selektiv in der weißen Milzpulpa an. Ein kleinerer Prozentsatz von ihnen (< 5 ) macht in den folgenden 48 h erstaunliche Veränderungen durch, die als Transformation bezeichnet wurden und deren Resultat «große pyroninophile Zellen» sind. Hier wurde beobachtet, daß RNS (Markierung mit $H_3$-Adenosin) vom Kern in das Plasma abströmt und dort in kurzer Zeit so verdünnt wird, daß sie nicht mehr nachweisbar ist; diese Zellen machen also eine Anzahl von rasch aufeinanderfolgenden Mitosen durch. Erstaunlicherweise folgt die Transformation einem Alles- oder Nichts-Gesetz, dem nur ein relativ kleiner Prozentsatz der Spenderlymphozyten unterliegt. Der häufige Einwand, daß

injizierte tritium markierte Aminosäuren lediglich von den Zellen des Wirtsorganismus reutilisiert würden, konnte an diesem System durch andersartige Kennzeichnung der transplantierten Zellen, z.B. mit Markier-Chromosomen, eindeutig widerlegt werden z.B können das Y-Chromosom in weiblichen Empfängertieren oder Rattenlymphozyten anhand ihrer Chromosomen in Mäusen zuverlässig wiedererkannt werden. An der Realität dieser Transformation der kleinen Lymphozyten kann also nicht gezweifelt werden.

*c) Die Reaktion vom verzögerten oder Tuberkulintypus (delayed hypersensitivity)* Seit den Versuchen von Chase und von Metaxas steht fest, daß die Tuberkulinsensibilität durch mononukleäre Zellen übertragen werden kann. Bei allen Versuchstieren erwiesen sich vitale Zellen dafür als notwendig Für den Menschen dagegen findet Lawrence eine Ausnahmestellung indem hier die Übertragung auch mit einem zellfreien «transfer factor» möglich sein soll. Sicher ist, daß sehr kleine Mengen der übertragenen Zellen für eine Sensibilisierung genügen die überwiegende Mehrzahl der Infiltratzellen stammt vom Wirt. Sie müssen auf irgendeine Art die spezifische Prägung von den injizierten Zellen übernehmen, wobei die Adsorption eines zytophilen Antikörpers eine Rolle spielen könnte.

Frappant ist jedenfalls die Ähnlichkeit der zellulären Veränderung in den regionären Lymphknoten mit bisher beschriebenen auch hier treten innerhalb von 24 h in den regionären Lymphknoten «große pyroninophile Zellen» auf, deren Plasma massenhaft Polyribosomen enthält. Auch diese Zellen teilen sich intensiv und geben in den folgenden Tagen zahlreiche Lymphozyten ab.

*d) In vitro-Kultur von Lymphozyten.* Bei Inkubation von Leukozyten aus peripherem Blut gehen die Granulozyten in wenigen Stunden zugrunde, während die Lymphozyten überleben. Sie können zur Umwandlung in große blastenähnliche Zellen veranlaßt werden durch verschiedene Agentien, von denen besonders das Phytohämagglutinin große praktische Bedeutung erlangt hat, da es heute fast in allen zytogenetischen Laboratorien regelmäßig angewendet wird. Der Wirkungsmechanismus dieser Stimulation ist unbekannt. Sehr aktuelles immunologisches Interesse ergab sich aus der Beobachtung daß auch spezifische Antigene diese Transformation auslösen können z.B. werden die Lymphozyten durch Diphtherie oder Tetanustoxoid, Tuberkulin und andere Antigene

spezifisch stimuliert, falls der Spender gegen diese Antigene vor immunisiert war Auch in einer Mischkultur der Lymphozyten von zwei verschiedenen Spendern erfolgt eine gegenseitige Stimulation. Bei Identität der H Antigene, also z. B bei eineiigen Zwillingen, bleibt dieser Effekt aus. Es wird weiter erforscht werden müssen, ob diese *in vitro*-Methode zuverlässig für den Nachweis von H Antigenen und von Sensibilisierungen eingesetzt werden kann.

Die stimulierten Zellen, die den großen pyroninophilen Zellen außerordentlich ähnlich sind, können sich weiter bis zu Plasma zellen differenzieren und Proteine sowie spezifische Antikörper sezernieren (s. später)

## 6 *Proteinsynthese*

Die einzige Proteingruppe, die zu den lymphoiden Geweben eine Beziehung hat, sind die Immunglobuline, auf die die weitere Besprechung deshalb eingeschränkt werden kann Als Immunglobuline oder $\gamma$-globuline sind die Träger der spezifischen Anti körper definiert. Diese Doppelnatur der Immunglobuline bringt den technischen Vorteil mit sich, daß dem Nachweis entweder Proteinreaktionen oder die Antikörperaktivität zugrunde gelegt werden können

Die zelluläre Basis der Antikörperbildung ist im letzten Jahr zehnt ebenfalls sehr intensiv bearbeitet worden Die daran beteilig ten Zellen wurden generell als «immunologisch kompetente Zellen» oder «Immunozyten» bezeichnet die Begriffe sind aber zu wenig präzis. Das Endstadium dieser Zellreihe, die Plasmazelle, ist wohl bekannt sie ist zytologisch und ultrastrukturell mit ihrem reich lichen Ergastoplasma (= endoplasmatischen Retikulum) der Prototyp einer sezernierenden Zelle. Weniger sicher sind aber die Kennt nisse über die Vorstufen der Plasmazellen und über die Frühphase der Antikörperbildung

Hier ist zurückzugreifen auf die frühen Versuche von Harris und von Ehrich die in den Lymphozyten aus dem Vas efferens von antigen-stimulierten Lymphknoten spezifische Antikörper fanden. Die Autoren sahen daher die Lymphozyten als Antikörperträger oder produzenten an. Diese sehr exakten Arbeiten gerieten später etwas in Vergessenheit, u. a. weil widersprechende Befunde an Ductus thoracicus-Lymphe erhoben wurden in der die antikörper

haltigen Lymphozyten jedoch stark verdünnt sind. Ferner schienen die Versuche von FAGRAEUS u. a. mit dem eindeutigen Nachweis der Antikörperproduktion durch Plasmazellen die Frage gelöst zu haben.

Die histologische und zytologische Untersuchung der zur Bildung humoraler Antikörper stimulierten Lymphknoten zeigt auffallend ähnliche Bilder wie bei der früher genau geschilderten graft versus host reaction. Die «transitional cells» von FAGRAEUS entsprechen den «großen pyroninophilen Zellen» durchaus. Aus ihrer lebhaften mitotischen Aktivität geht wieder eine große Anzahl kleiner Lymphozyten hervor, die in das Vas efferens des Lymphknotens abgegeben werden. Es scheint sehr plausibel, daß sie die von HARRIS und EHRICH gefundenen antikörperhaltigen Lymphozyten sind. Neue autoradiographische Befunde bestätigen übrigens den großen Lymphozyten als Vorläufer der Plasmazellen. Die scheinbar widersprechenden Befunde sind also ohne weiteres vereinbar, da sie aus verschiedenen Phasen der gleichen Entwicklung stammen. Auch hier deuten wieder verschiedene experimentelle Befunde darauf hin, daß initial die kleinen Lymphozyten mit dem Antigen reagieren und die Entwicklung antikörperbildender Zellen über die Zwischenstufe eines sich teilenden Vorläufers einleiten. Dem Lymphknoten kommt dabei eine zentrale Bedeutung zu. In seinen Maschen werden die relativ wenigen reaktionsfähigen Lymphozyten abgefangen und in engen Kontakt mit dem Antigen gebracht.

*Antikörperbildung durch die Einzelzelle.* In äußerst minutiösen Versuchen mit dem Mikromanipulator konnte NOSSAL die Bildung von Antikörpern durch individuelle Einzelzellen studieren. Bei Versuchstieren, die mit zwei Antigenen zugleich immunisiert wurden, konnte er zeigen, daß jede Einzelzelle nur einen Antikörper bestimmter Spezifität bildet. Demnach wurde das Synthesesystem der Einzelzelle auf ein und nur ein bestimmtes Antigen eingestellt und spezialisiert. Andere Autoren fanden allerdings mit prinzipiell derselben Technik, aber mit Virus-Antigenen, entgegengesetzte Befunde, d. h. die simultane Bildung von zwei Antikörpern durch eine individuelle Zelle.

Die Zytologie der immunglobulinbildenden Zellen ist vor allem mit Hilfe fluoreszierender Antikörper ausführlich studiert worden: tierische Antikörper verschiedener Spezifität werden mit Fluoreszein markiert und mit den zu prüfenden Zellen im Ausstrich

spezifisch stimuliert, falls der Spender gegen diese Antigene vorimmunisiert war. Auch in einer Mischkultur der Lymphozyten von zwei verschiedenen Spendern erfolgt eine gegenseitige Stimulation. Bei Identität der H-Antigene, also z. B. bei eineiigen Zwillingen, bleibt dieser Effekt aus. Es wird weiter erforscht werden müssen, ob diese *in vitro*-Methode zuverlässig für den Nachweis von H-Antigenen und von Sensibilisierungen eingesetzt werden kann.

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dung angeregt werden kann. Bei Patienten mit agammaglobulinämischem Antikörpermangelsyndrom erfolgt gar keine Reaktion auf spezifische Antigene, aber eine qualitativ und quantitativ völlig normale Transformation unter der Wirkung von Phytohämagglutinin dieses Agens stimuliert die lymphoiden Zellen der Patienten bis zur Umwandlung in reife Plasmazellen, die sogar Immunglobuline synthetisieren können. Dies spricht dafür daß die Störung bei der Agammaglobulinämie nicht bei der Bildung der Plasmazellreihe an sich liegt, sondern auf dem Nicht Ansprechen auf den physiologischen Antigenreiz beruht. Da die immunologischen Reaktionen vom verzögerten Typ erhalten sind, ist hier nur das bursaabhängige immunologische System gestört, nicht aber das thymusabhängige. Bei der bedeutend schwereren, ebenfalls hereditären Störung des hypogammaglobulinämischen Antikörpermangelsyndroms mit schwerer Lymphopenie konnten wir nachweisen, daß auch die Phytohämagglutinin-Stimulation der Lymphozyten erfolglos bleibt. Dementsprechend versagen auch die übrigen Funktionen der Lymphozyten (delayed hypersensitivity Transplantations- und Histokompatibilitäts-Reaktionen)

## *7 Zusammenfassung der experimentellen Befunde*

Die Fülle der vorliegenden experimentellen Ergebnisse erschwert den Überblick. Einige allgemein gültige Tatsachen über die Bedeutung der Lymphozyten in der immunologischen Abwehr können jedoch mit Sicherheit festgehalten werden die Ansicht der klassischen morphologischen Hämatologie, daß der Lymphozyt die Endphase einer Entwicklung darstelle, muß revidiert werden. Er ist eine ruhende Zelle, die jederzeit durch adäquate Stimulation wieder in lebhafte Tätigkeit gesetzt werden kann. Er ist ferner eine ubiquitär in sämtlichen Geweben vorkommende und aktiv bewegliche Zelle. Ferner ist an einer ständigen Rezirkulation und an einer außerordentlich langen Lebensdauer von schätzungsweise 80 % der kleinen Lymphozyten nicht mehr zu zweifeln. Schließlich ist aus der vorangehenden Schilderung der Einzelbefunde bei verschiedenen experimentellen Anordnungen eine Monotonie der Reaktionsarten unverkennbar der ruhende kleine Lymphozyt wird durch den Antigenstimulus in zwei Phasen aktiviert zunächst geht er in eine große pyroninophile Zelle über die dann eine lebhafte

mitotische Aktivität aufnimmt und eine große Anzahl Tochterzellen liefert.

Simplifiziert, verallgemeinert und ein wenig spekulativ kann die Rolle der Lymphozyten folgendermaßen geschildert werden: sie sind die Träger der genetischen Information «im Taschenbuchformat» und kontrollieren ununterbrochen zirkulierend sämtliche Gebiete des Körpers. Abweichungen der antigenetischen Struktur von ihrem genetischen Pflichtenheft bilden den adäquaten Stimulus, der sie aus ihrer Inaktivität erweckt. Sie sind also zur Unterscheidung zwischen körpereigenen und körperfremden Strukturen fähig (self und not-self nach Medawar); auf diese Art halten sie die «immunologische Homöostase» aufrecht. Die Aktivität eines stimulierten Lymphozyten verläuft in drei Phasen, die mit dem Ablauf eines Reflexbogens vergleichbar sind: im afferenten Schenkel werden Informationen über das fremde Antigen oder dieses selbst zu einem Zentrum transportiert. Hier (im regionären Lymphknoten oder in der Milz) erfolgt die zentrale Auswertung der Information und die Organisierung der Abwehrreaktion mit der eben geschilderten Bildung teilungsfähiger Zellen mit zahlreicher Progenie von antigen-geprägten Tochterzellen. Der efferente Schenkel schließlich umfaßt die Immigration dieser aktivierten Zellen in die von fremdem Antigen durchsetzten Gebiete oder die Abgabe humoraler Antikörper an den Blutstrom.

Zahlreiche immunologische, ontologische und phylogenetische Befunde sprechen dafür, daß zwei weitgehend voneinander unabhängige immunologische Systeme bestehen, deren frühembryonale Induktion einerseits vom Thymus, andererseits von der Bursa Fabricii oder ähnlichen Organen (beim Menschen den Tonsillen?) gesteuert wird. Das erste System liefert die kleinen Lymphozyten und vermittelt Reaktionen vom verzögerten Typus, Transplantations- und Histokompatibilitätsimmunität; das bursa-abhängige System liefert die großen Lymphozyten, die sich nach Stimulation zu Plasmazellen entwickeln, in denen die humoralen Antikörper gebildet werden.

Von den Theorien der Antikörperbildung hat vor allem Burnet's clonal selection-Theorie unser Denken auch in klinischer Beziehung und über die Immunologie hinausgehend stark beeinflußt. Sie ist mit einigen Modifikationen imstande, fast sämtliche experimentelle Befunde zwanglos zu erklären. Im Rahmen dieser Übersicht kann aber nicht näher darauf eingegangen werden.

## 8 Klinische Aspekte

Von den humanmedizinisch beobachteten Störungen immunbiologischer Mechanismen sind die angeborenen und vererbten Defekte als «Naturexperimente» besonders aufschlußreich. Alle aus der phylogenetischen Entwicklung voraussehbaren Störungen wur den klinisch tatsächlich beobachtet. Die Ausfallserscheinungen entsprechen, soweit sie genauer erforscht wurden den erwarteten. Wir hatten Gelegenheit, einige Fälle mit der schweren rezessiv autosomal vererbten Form der Agammaglobulinämie mit schwerer Lymphopenie zu untersuchen. Die davon betroffenen Kinder erkranken bereits in den ersten Lebensmonaten an rezidivierenden Infekten und schwerer Diarrhöe, die zu einer rasch progredienten Dystrophie führt. Fast regelmäßig führen pulmonale Infekte im ersten Lebensjahr zum Tode. Ausführliche Prüfungen bewiesen den vollständigen Ausfall der Fähigkeiten zur humoralen Antikörperbildung, zur Abstoßung von Transplantaten und zur Reaktion vom verzögerten Typus. Die fast völlige Aplasie und fehlende Differenzierung des Thymus deutet auf ein Ausbleiben der frühfoetalen Induktion des immunbiologisch aktiven Gewebes hin. Therapeutische Versuche werden daher darauf ausgerichtet sein, diese Entwicklung nachzuholen oder zu induzieren. Zur Vermeidung einer graft versus host reaction kommt dafür nur die Implantation foetaler Gewebe in Frage. Bei zwei Fällen konnten wir tatsächlich einen signifikanten Anstieg der Lymphozyten beobachten, der für die Richtigkeit dieser Hypothese spricht beim zweiten Fall normalisierten sich die Lymphozytenzahlen während mehreren Monaten. Trotzdem erwiesen sich diese Zellen aber als qualitativ minderwertig und funktionell insuffizient, wie aus allen entsprechenden Prüfungen hervorging Die Ersatztherapie durch Nachholung der embryonalen Entwicklung war also erfolgreich, aber dem entwickelten immunologischen Gewebe fehlt das wichtige Charakteristikum der Reaktionsfähigkeit. Wie so häufig sind auch hier bei der Lösung einer Frage sieben neue aufgetaucht. Wir sind aber überzeugt, daß aus der weiteren Erforschung dieser kongenitalen Entwicklungsstörungen wesentliche Aufschlüsse zu erwarten sein werden.

Auf vielen weiteren Gebieten wird die genaue Kenntnis und Auswertung der tierexperimentellen Immunologie zweifellos von großer Bedeutung werden sie ist absolute Voraussetzung für die Durchführung von Haut und Organtransplantationen sie ist von

wesentlicher Bedeutung für die zytostatische und immunodepressive Therapie; schließlich bestehen Hinweise für wesentliche Zusammenhänge mit der Karzinogenese, bzw. mit dem Schutz gegen karzinogene Auswirkungen. Die klinische Immunpathologie schließlich hat sich seit längerer Zeit zu einem großen eigenen Gebiet entwickelt. In allen diesen Bereichen wird zukünftig ein vermehrtes Zusammenwirken zwischen Hämatologen und Immunologen nötig sein.


### *Zusammenfassung*

Übersicht über neuere Befunde an Lymphozyten: Lebensdauer, Rezirkulation, Transformation in mitotisch aktive Zellen. Diese Tatsachen werden zu Funktionen der Lymphozyten bei der Erkennung und Eliminierung körperfremder Antigene in Beziehung gesetzt. Der Lymphozyt ist demnach der Wächter über die »immunologische Homöostase« des Organismus.

### *Summary*

New knowledge concerning the lymphocytes is reviewed: survival, recirculation, transformation into mitotically active cells. The relation of these data to the function of lymphocytes in recognition and elimination of foreign antigens is discussed. The lymphocyte is thus the "guardian of immunological homeostasis" in the organism.

### *Résumé*

Quelques nouveaux résultats des recherches sur les lymphocytes sont passés en revue: résultats concernant leur temps de vie, leur récirculation, leur transformation en cellules se multipliant par mitose. Ces résultats sont mis en relation avec les fonctions que les lymphocytes ont lors du repérage et de l'élimination d'antigènes étrangers. Ainsi le lymphocyte serait le gardien de "l'homéostase immunologique" de l'organisme.


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C. R. 10e Congr. Soc. europ. Hémat., Strasbourg 1965 Acta haemat. 36: 63–88 (1966)

Centre de Recherches sur les Macromolécules, Strasbourg

# Configurations des macromolécules en solution
## Méthodes d'étude

Ch. SADRON

## I. Les Macromolécules

### *Les macromolécules et la biophysique moléculaire*

L'un des caractères importants – et peut-être l'un des plus riches de conséquences – de l'évolution de nos connaissances sur la structure moléculaire de la matière s'est manifesté vers la fin du premier tiers de notre siècle par l'apparition de la physicochimie macromoléculaire ainsi que par les applications de cette science à l'interprétation des phénomènes biologiques fondamentaux.

D'une part en effet les physicochimistes – et c'est là que s'est manifesté particulièrement le mérite de STAUDINGER et de son école – ont commencé à comprendre clairement vers 1930 qu'il pouvait exister – et mieux encore que l'on pouvait synthétiser – des molécules géantes dont la masse peut atteindre jusqu'à un million de fois la masse de la molécule d'eau, renfermant de ce fait des milliers, des centaines de milliers et peut-être même des millions d'atomes et dont les dimensions sont presque suffisantes pour tomber dans le domaine du pouvoir séparateur des bons microscopes optiques. C'est à STAUDINGER que l'on doit, pour les désigner l'appellation de macromolécule, terme qui a connu depuis le succès que l'on sait.

D'autre part – et là nous nous reportons aux célèbres travaux de SVEDBERG sur la détermination des masses des protéines par ultracentrifugation – vers la même époque (1933) il fut reconnu que les substances protéiques, dont on savait déjà que le poids moléculaire devait être élevé se comportaient également comme des molécules géantes, s'apparentant ainsi aux macromolécules de STAUDINGER (fig 1) Depuis cette époque les biologistes analysant

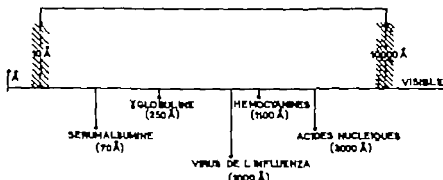



*Fig. 1* Le domaine macromoléculaire.

de manière de plus en plus subtile la structure des tissus ont reconnu que l'un des caractères permanents de la matière vivante était de contenir à côté de l'eau et des sels minéraux des macromolécules organiques, telles les protéines et les acides nucléiques dont la présence apparaît ainsi comme indispensable à la manifestation de la vie.

Aujourd'hui l'étude des propriétés de ces macromolécules biologiques constitue un point de rencontre entre biologistes et physicochimistes et ainsi la biophysique (ou biophysicochimie) moléculaire apparaît elle comme une science en plein essor née – on vient de le voir – de ce que l'évolution de la physicochimie allant du simple au complexe et de ce que l'évolution de la biologie allant du complexe au simple ont débouché sur un territoire commun, celui de la complexité macromoléculaire.

Il ne faut pas croire pour autant que, du moins dans l'état actuel de nos connaissances, macromolécules de synthèse et macromolécules biologiques (celles-ci étant restreintes aux espèces «nobles» telles les protéines globulaires et les acides nucléiques) ne se distinguent que par la manière dont elles ont été obtenues: synthèse organique pour les premières, analyse des tissus ou synthèse enzymatique pour les secondes.

Il faut souligner que protéines et acides nucléiques présentent des propriétés – catalyse enzymatique ou guides de replication (templates en anglais) pour les secondes – que l'on ne retrouve aucunement dans les macromolécules de synthèse que nous savons fabriquer de nos jours (à l'exception du fait que certains oligonu

cléotides de synthèse abiotique peuvent induire – mais en présence toutefois d un enzyme – certaines polymérisations) De ce fait le dualisme entre l inanimé et le vivant semble se retrouver à l'échelle élémentaire des macromolécules. Et l on comprend avec quelle ardeur passionnée les physicochimistes s attachent à réduire ce hiatus irritant dont la disparition laissera présager la complète compréhension du biologique par le physicochimique, ce qui ouvre à la Science des perspectives exaltantes.

Mais, pour comprendre les mécanismes que les macromolécules biologiques peuvent faire jouer il est d abord indispensable de connaître leur structure puisque c est la structure qui permet la fonction. En particulier nous ne sommes pas étonnés de constater qu aux différences de fonction que nous venons de relever entre macromolécules de synthèse et macromolécules biologiques vont correspondre entre celles-ci des différences entre des structures qui – bien que comparables – sont autrement plus complexes dans le second cas que dans le premier Rappelons quelques données essentielles.

### *Les macromolécules en chaîne*

a) *Les Polymères* La physicochimie macromoléculaire nous a appris que l on pouvait, par diverses méthodes qu il n y a pas lieu d énumérer ici, effectuer la synthèse de longues chaînes moléculaires comparables à des chapelets dont les boules seraient formées par un groupe moléculaire contenant un petit nombre d atomes. Chacune de ces «boules» est articulée à ses deux voisines par des liaisons chimiques très solides (liaisons covalentes) mais qui permettent cependant le jeu de mouvements relatifs. Ainsi le chapelet est-il à la fois stable et plus ou moins flexible, selon la nature chimique des motifs dont la répétition le constitue (fig 2)

C'est cette flexibilité qui est la cause des propriétés très particulières des hauts polymères (visco-élasticité, caoutchouc, fibres, etc...)

Quand elle est en solution la chaîne moléculaire est soumise au mouvement brownien qui fait onduler sans cesse le filament monocaténaire. On conçoit que si celui-ci est très long et très flexible ses configurations les plus probables se présenteraient, si l'on pouvait les observer comme une pelote embrouillée la «pelote statistique» que l on peut, par la pensée, inclure dans un volume fini sphéroïdal (fig 3)

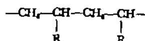
## Exemples de molécules en chaîne

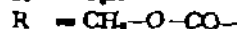
### *1 Polymères*

1 Chaînes non substituées
Paraffines (Polythènes)

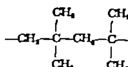


2. Chaînes monosubstituées
Dérivés polyvinyliques



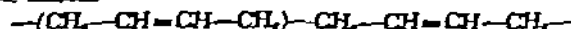
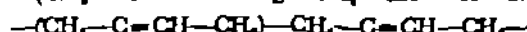
Exemples:
Chlorure de polyvinyle 
Polystyrols 
Esters polyacryliques 
Orlon (acrylonitrile) 

3. Chaînes disubstituées
Polyisobutènes (Oppanol)



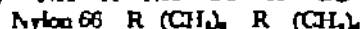
4 Chaînes tétrasubstituées
Téflon



5. Chaînes contenant des double-liaisons
Polybutadiènes 
Polyisoprènes (Caoutchoucs) 
 

### *2. Polycondensats*

1 Polyamides



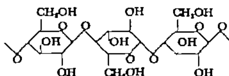
2. Polyesters





*3 Molécules en chaînes naturelles*

1 Cellulose




2. Polypeptides




3. Acides nucléiques

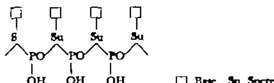



*Fig* 2a et 2b Quelques exemplaires de hauts polymères.

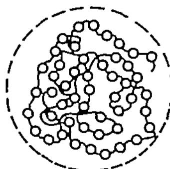


*Fig* 3. Schéma de pelote statistique.

Mais il peut arriver aussi que dans certaines conditions de solvant et de température il s'exerce entre les motifs de la chaîne des forces d'attraction suffisantes pour que toute la macromolécule soit figée en une configuration unique.

L'expérience montre alors que celle-ci se présente sous la forme d'une longue hélice (hélice α de Pauling et Corey) Un exemple particulièrement important est celui des polypeptides, dont le motif élémentaire est $—H_2N—CH—CO—$ (fig 4)



R

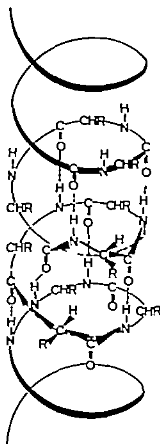



*Fig. 4.* Schéma de l'hélice de PAULING et COREY
Rayon 1,81 Å Pas de l'hélice 5,44 Å Translation par résidu 1,47 Å
Rotation entre 2 résidus 97°2 Il y 3,6 motifs par pas (hélice 3.6)

Le long filament hélicoïdal est encore légèrement flexible et il présente de ce fait des configurations sinueuses mais il est en général trop rigide pour ressembler à une pelote statistique.

On peut d'ailleurs passer d'une hélice à une pelote statistique en rompant par l'action d'agents physiques ou chimiques les interactions entre les motifs de la chaîne. La macromolécule perd son hélicité et redevient le simple filament souple qui forme alors une pelote statistique désordonnée. Dans certains cas ce phénomène est brutal et comparable à une véritable fusion.

b) *Protéines et acides nucléiques*

*Structures primaire, secondaire et tertiaire.* Cela étant dit un fait fondamental est que, tout comme les macromolécules de synthèse, les macromolécules biologiques – protéines, acides nucléiques – sont

essentiellement des molécules en chaîne. De ce point de vue on peut donc dire que les processus de biosynthèse et les processus de synthèse abiotique sont étroitement apparentés.

Mais aussitôt des différences importantes apparaissent dans l'ordre des complexités relatives.

La chaîne protéique peut renfermer des motifs d'espèces différentes (ici les résidus d'acides aminés) et le nombre de ces dernières peut s'élever jusqu'à une vingtaine, alors que la synthèse abiotique ne donne guère que des polypeptides contenant au plus deux ou trois espèces différentes d'aminoacides.

On sait maintenant que les caractères spécifiques de la protéine dépendent non seulement de sa composition, mais aussi de l'ordre d'enchaînement des motifs dans la chaîne. C'est-à-dire de la *structure primaire* de celle-ci.

De plus – sans doute à cause de la complication de cette structure primaire (et peut-être aussi à cause du processus de biosynthèse mis en œuvre dans l'organisme vivant) – la chaîne protéique ne se présente pas comme une hélice α régulière elle s'enroule par portions, mais reste linéaire en quelques autres, et cela dans des proportions variables selon la nature de la protéine (près de 100 % d'hélicité dans les globines, près de 0 % dans la ribonucléase) C'est la *structure secondaire*

Enfin, même si l'hélicité est totale, le boudin hélicoïdal est tordu sur lui même en volvulations tourmentées c'est la *structure tertiaire* (fig 5) La protéine se présente donc en gros comme une



Fig 5 Forme de la molécule de myoglobine d'après Kendrew L'espece de tube contourné est une hélice α.

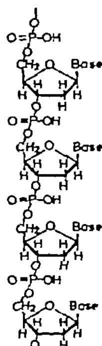
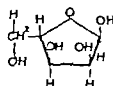



*Fig. 6.* Chaîne de polydeoxyribonucléotide.



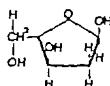
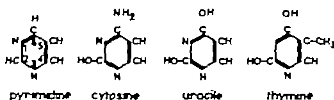




*Fig. 7*




BASES PYRIMIDIQUES

BASES PURIQUES

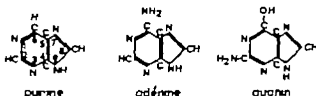



*Fig. 8.* Les bases constitutives du DNA.
Thymine et cytosine: bases pyrimidiques.
Adénine et Guanine: bases puriques.

particule souvent globulaire dont la description morphologique est difficile. Il faut aussitôt ajouter que cette morphologie dépend essentiellement du milieu solvant (force ionique, acidité, température) dans lequel la molécule se trouve dispersée. Donc en général les fonctions – qui dépendent de la configuration – dépendent aussi de ces facteurs.

Le cas des acides nucléiques est un peu plus simple, au moins en apparence. Ce sont des chaînes de phosphodiesters (fig 6) de sucres (ribose ou désoxyribose [fig 7]) dont un des sites est substitué par des bases puriques ou pyrimidiques d'un petit nombre d'espèces (deux purines et deux pyrimidiques) (fig 8)

Ces chaînes se présentent parfois (acides ribonucléiques) sous forme de pelotes statistiques avec des zônes de repliement plus ou moins régulier Dans le cas de l'acide désoxyribonucléique, la forme naturelle est un filament bicaténaire, à appariement complémentaire, selon CRICK et WATSON (figs. 9 et 10)

Là encore on distingue une structure primaire répartition des divers nucléotides selon des séquences déterminées dont on sait qu'elles représentent le code de l'information génétique une structure secondaire qui correspond à l'enroulement hélicoïdal une structure tertiaire qui correspond à la configuration (ou à la statistique des configurations) du filament nucléique, notion particulièrement nette dans le cas des acides ribonucléiques.

*Intérêt de l'étude morphologique* Pour les unes comme pour les autres de ces macromolécules les configurations secondaire et tertiaire – la structure primaire étant supposée connue – sont sous la

| | | | | | |
|---|---|---|---|---|---|
| phosphate – sucre | adénine | | thymine | sucre | phosphate |
| phosphate | sucre | cytosine | guanine | sucre | phosphate |
| phosphate | sucre | guanine | cytosine | sucre | phosphate |
| phosphate | sucre | thymine | adénine | sucre | phosphate |
| phosphate | sucre | thymine | adénine | sucre | phosphate |

*Fig* 9. Schéma de l'appariement entre deux filaments désoxyribonucléiques complémentaires.

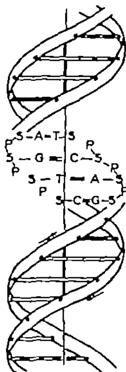



*Fig. 10* Schéma d'enroulement en double hélice des filaments polynucléotidiques complémentaires.

dépendance des interactions plus ou moins intenses s'exerçant entre les constituants de la chaîne. Ces interactions sont de diverses origines ionique, dipolaire, quantique, et la physicochimie macromoléculaire moderne les soupçonne à juste titre d'être parmi les facteurs les plus importants qui provoquent les propriétés si particulières des macromolécules biologiques Il s'ensuit donc que l'étude complète de celles-ci pose de difficiles problèmes d'électrochimie et de physique quantique. Mais tout compte fait, toutes ces interactions conditionnent les configurations macromoléculaires de telle sorte que l'étude de celles-ci permettra de remonter aux propriétés fondamentales intrinsèques de la molécule. D'ailleurs l'étude des configurations – de la morphologie si l'on préfère – présente un intérêt direct puisque l'on sait que la topologie de certains groupes moléculaires portés par une protéine – certaines enzymes par exemple – donne à leur ensemble non seulement leur activité réactionnelle, mais encore le caractère spécifique de celle-ci. Enfin les réactions auxquelles prennent part les macromolécules seront très fré-

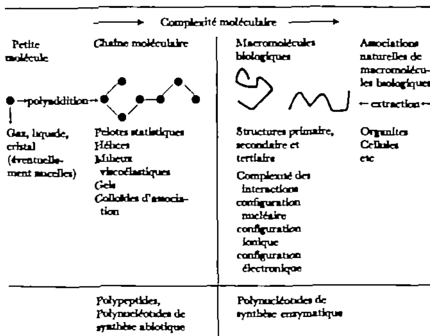



Fig. 11

quemment suivies par les modifications qu'elles apportent à la morphologie de celles-ci (masses, configurations). Pour tout ces raisons – parmi tant d'autres – on conçoit que l'étude morphologique des macromolécules biologiques en fonction des conditions dans lesquelles se trouvent leurs solutions constitue l'une des activités principales de ce que nous avons appelé dès le début la biophysicochimie macromoléculaire et dont nous pouvons maintenant, pour conclure ce chapitre introductif, illustrer la situation relative à celle de la biologie moléculaire et à la physicochimie classique par le schéma de la fig. 11.

## II. Méthodes d'étude des configurations

### *Position du problème*

Le problème que nous posons est alors celui-ci : ayant préparé – ou extrait d'un tissu – une espèce donnée de macromolécules que l'on a mise en solution il s'agit de déterminer la masse de cette espèce macromoléculaire ainsi que ses structures primaire, secondaire et

Nous laisserons ici de côté l étude générale de la polydispersité nous y reviendrons seulement dans le cas où, exceptionnellement et par sa nature particulière, la méthode employée (par exemple la sédimentation par ultracentrifugation) permet de mesurer la valeur de $\varphi_i$ pour chaque type des macromolécules existant dans le mélange.

Dans ce qui suit nous considérerons donc – sauf mention particulière – que nous avons à faire – par exemple après un fractionnement convenable – à une solution monodisperse

Enfin encore une remarque préalable et de signification très générale. Dans une solution de concentration donnée les macromolécules interagissent les unes avec les autres d autant plus fortement que leur nombre par unité de volume est plus élevé. Il en résulte que, d une façon très générale et pour des raisons diverses que *nous n examinerons pas ici, la grandeur $\varphi$ dans l équation (2) dé*pend de $c$ Cet effet de la concentration est souvent très difficile à calculer et l on préfère s en débarrasser par un moyen expérimental simple. Considérons en effet l équation (2) à partir de (1) on peut la mettre sous la forme

$$\Phi = \frac{c}{M} \Lambda \varphi \quad \text{ou encore} \quad \frac{\Phi}{c} = \frac{\Lambda}{M} \varphi \tag{3}$$

En mesurant $\Phi$ à diverses concentrations c et en prenant la valeur limite de $\Phi/c$ pour $c = 0$ l équation (3) permet de calculer la valeur limite de $\varphi$ (que nous désignerons par $\varphi_0$) pour la concentration nulle.

Cette grandeur $\varphi_0$, que la série d expériences permet de déterminer est alors bien caractéristique de la macromolécule elle correspond au cas idéal où celle-ci est seule dans un volume de solvant infini.

C est cette valeur de $\varphi_0$ – dont la mesure encore une fois demande toute une série de déterminations à concentrations décroissantes suivies d une extrapolation à concentration nulle – dont nous nous occuperons dorénavant

*Cela posé on classe les diverses méthodes donnant ($\Phi$) ou $\varphi_0$ en* deux grandes catégories

les méthodes hydrodynamiques

et les méthodes de diffusion électromagnétique.

Nous allons en donner ci-dessous, l énumération en nous bornant exclusivement à leur principe sans pouvoir entrer – le cadre de cet exposé nous en empêche dans aucun détail technique.

### *Mesure de φ : les méthodes hydrodynamiques*

a) *Les coefficients de frottement visqueux*

Supposons que la macromolécule se déplace relativement au liquide extérieur avec un mouvement de translation de vitesse v. Dans ce mouvement relatif elle subit, de la part du liquide, un frottement visqueux proportionnel à v et à la viscosité $\eta_0$ du liquide

$$F = f \eta_0 v$$

On conçoit que f ne dépende que de la forme et des dimensions de la molécule.

De même si l'on fait tourner la molécule autour d'un de ses axes avec la vitesse angulaire ω elle subira un couple de frottement

$$\Gamma = C \eta_0 \omega$$

De même que f, C dépend de la forme et des dimensions de la molécule.

Il est donc important – dans le cadre de nos préoccupations – de mesurer les coefficients unitaires de frottement de translation f et de rotation C.

### *Mesure de f*

A cause du mouvement brownien de translation la molécule se déplace irrégulièrement en tous sens dans la solution et cela d'autant plus énergiquement que la température est plus élevée. C'est ce qui fait que les molécules peuvent diffuser hors d'une solution vers le solvant libre. On peut, par l'expérience, mesurer dans ce cas un coefficient de diffusion de translation Δ qui est lié à f par la relation

$$\Delta = \frac{kT}{f \eta_0} \qquad (4)$$

T : température absolue,
k : constante de Boltzman ($k = 1{,}38 \cdot 10^{-16}$ erg)
Comme on connait $\eta_0$ la mesure de Δ donne f.

On peut aussi appliquer à la molécule une force F qui la fait se déplacer avec la vitesse v. On a alors

$$F = f \eta_0 v \qquad (5)$$

Donc on peut tirer M de la mesure simultanée de $s$ et de $\Delta$. C'est la méthode de SVEDBERG.

*Remarque 2* L'emploi de la méthode de centrifugation a pris une grande extension dans les récentes années. L'une des raisons en est que parmi toutes les techniques en cours, elle est sans doute celle qui demande la mise en œuvre de quantités de substances les plus faibles (quelques fractions de mg) ce qui a une grosse importance en biologie. Mais surtout son intérêt se manifeste pleinement dans le cas des solutions polydispersées.

1 Si la solution contient des particules auxquelles correspondent des valeurs différentes de $s$ – donc migrant avec des vitesses différentes – la répartition de la concentration de la solution au bout d'un temps t permet de tracer la répartition des valeurs de $s$. Par exemple on peut ainsi établir le pourcentage $g(s)\,\Delta s$ des macromolécules présentant des constantes de sédimentation comprises entre $s$ et $s + \Delta s$ (fig 12) On dispose ainsi d'une méthode permettant de déterminer si une solution contient des macromolécules présentant des valeurs de M/f différentes, ce qui rend, en biologie, les plus grands services.

2. D'après les équations (6) et (7) on voit que, dans un solvant de densité $\varrho$ telle que $1 - V_{sp}\varrho = 0$ il n'y a pas de vitesse de sédimentation tandis que celle-ci est positive ou négative selon le signe de ce binôme. Donc si l'on dispose d'un mélange de particules dont

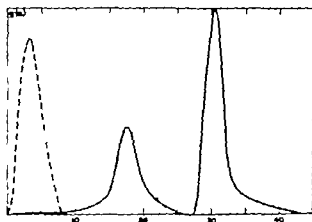



*Fig 12* Exemple de l'analyse d'un mélange par sédimentation. Distribution de g( ) dans une solution de RNA ribosomal montrant l'existence dans le mélange de deux espèces différentes ( = 18 et = 30 En pointillé g( ) du produit de la dégradation enzymatique de ce mélange (TIMASHEFF WITZ et LUZZATI, Biophys. J (1961) 525)

le volume spécifique partiel est différent on voit que l'ultracentrifugation permet – même si M/f est le même pour toutes ces particules – de séparer les molécules selon leurs densités. C'est là le principe de la méthode de Meselson

*Mesure de φ Méthodes de diffusion du champ électromagnétique*

Considérons un faisceau de lumière parallèle, polarisé verticalement (cette condition n'est pas indispensable, mais est utile pour la simplification de l'exposé) traversant un liquide transparent pour la longueur d'onde λ de rayonnement employé (fig 13)

On sait que les molécules du liquide, excitées par le rayonnement incident, réémettent – on dit «diffusent» – dans toutes les directions de la lumière de même longueur d'onde. Si maintenant on introduit dans le liquide, jouant alors le rôle d'un solvant, des macromolécules d'indice de réfraction différent (et qui n'absorbent pas la lumière monochromatique employée) on constate que l'intensité de la lumière diffusée devient plus grande.

Désignons par I (θ) cette augmentation de l'intensité diffusée dans la direction définie par l'angle θ

On démontre que I (θ) dépend de la concentration c, de l'indice de réfraction de la solution, de la masse moléculaire du soluté des dimensions et de la forme des macromolécules dissoutes.

On peut alors, en mesurant par un dispositif approprié, I (θ)/c en fonction de θ déterminer par extrapolation la valeur de $I_1(\theta)/c$ pour c = 0 La théorie montre que l'on peut, de cette grandeur tirer

la valeur M de la masse des molécules dissoutes,

la grandeur R de leur rayon de giration et cela aussi compliquée que soit la forme de la macromolécule, à condition cependant

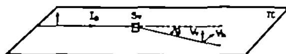



*Fig 13.* $I_0$ faisceau incident polarisé verticalement.
V volume de matière diffusant
$V_v$ intensité du faisceau diffusé (composante verticale)
$V_h$ intensité du faisceau diffusé (composante horizontale)

---

Si la macromolécule peut être considérée comme un ensemble de N points matériels $A_i$ répartis autour du centre de gravité G, le rayon de giration est défini par R = … / N où … = $A_iG$.

que les dimensions macromoléculaires soient largement supérieures au vingtième de la longueur d onde incidente.

De plus si les particules ont des dimensions au moins de l ordre de grandeur de $\lambda$, alors la loi de variation de $I(\theta)$ en fonction de $\theta$ permet de discerner si la particule est comparable à une sphère, un ellipsoïde de révolution un bâtonnet ou un long filament flexible, ce qui est un renseignement extrêmement précieux. Et même dans les deux derniers cas, et dans des conditions favorables, la méthode permet d évaluer la masse du bâtonnet ou du filament par unité de longueur et même encore d avoir une idée des configurations du filament (zig zag de longs bâtonnets, étoiles, filament sinueux de flexibilité plus ou moins grande)

Ainsi voit-on que la méthode de la diffusion de la lumière donne des possibilités très intéressantes pour la détermination de la morphologie des macromolécules en solution.

Cependant – revenons y – elle a l'inconvénient de ne donner autre chose que la masse lorsque les particules ont des dimensions inférieures à environ $\lambda/20$ Ainsi avec la lumière verte du mercure ($\lambda = 546\ m\mu$) les macromolécules de dimensions inférieures à 250 Å se conduisent comme des points matériels. On ne peut mesurer leur rayon de giration. On peut échapper à la difficulté en employant un rayonnement incident de longueur d onde plus faible. C'est ainsi qu on a pu utiliser la diffusion centrale des rayons X (ne pas confondre avec la diffraction de BRAGG) Cette méthode a permis d obtenir de bons résultats dans la mesure du rayon de giration des protéines.

### *La détermination des masses formes et dimensions*

1 *Récapitulation*

En résumé, de l énumération qui précède on tire la conclusion que – une solution monodisperse étant donnée – on peut déterminer les caractères macromoléculaires suivants

a) La masse (détermination directe par équilibre de sédimentation, diffusion de la lumière, éventuellement par pression osmotique)

b) Le coefficient f de frottement visqueux de translation (constante J de diffusion de translation)

Le coefficient C de frottement visqueux de rotation (constante de diffusion B de rotation)

Le rayon de giration (diffusion de la lumière ou des rayons X)

c) On doit ajouter à cela les caractères hybrides (c'est-à-dire dépendant de la masse et de la forme) suivants
la constante de sédimentation s, dépendant de M et de f
la viscosité intrinsèque dépendant de F et de M.

Dans ce cas, en combinant la mesure de f et de $d$ on obtient M. Ou encore, connaissant M déterminé par une mesure directe, on peut calculer f à partir de s.

2. *Détermination de la forme et des dimensions*

Disposant ainsi des grandeurs morphologiques telles que f, C, ($\eta$) s et R il s'agit à partir de celles-ci de déterminer la forme et les dimensions de la macromolécule en solution.

Supposons que, par un moyen quelconque, nous connaissions la forme de la macromolécule et que cette forme soit assimilable à un modèle simple sphère, bâtonnet, disque, ellipsoïde de révolution filament ou pelote statistique, alors dans ce cas il est possible de calculer les dimensions correspondantes.

Cela est évident dans le cas du rayon de giration R, grandeur géométrique, pour lequel un calcul simple donne les résultats suivants

| | |
|---|---|
| bâtonnet de longueur L | $R^2 = \frac{L^2}{12}$ |
| sphère de rayon L | $R^2 = \frac{3}{5} L^2$ |
| ellipsoïde de révolution (a, b c étant les trois paramètres) | $R^2 = \frac{1}{5}(a^2+b^2+c^2)$ |
| pelote statistique gaussienne ($\overline{L^2}$ étant le carré moyen de la distance entre les extrémités de la chaîne) | $R^2 = \frac{\overline{L^2}}{6}$ |

Les choses ne sont pas aussi simples pour l'utilisation des autres grandeurs, qui sont des grandeurs physiques qui ne pourront être liées aux dimensions que par le moyen d'une théorie physique appropriée.

Dans le cas où nous nous sommes placés ici (petite particule solide de forme simple) et en admettant que les lois de l'hydrodynamique des fluides continus restent valables à cette échelle (ce qui cesse d'être exact quand les dimensions de la particule sont de l'ordre de celles des molécules de solvant) on peut alors établir les

relations cherchées. Celles-ci sont assez compliquées et nous donnerons ici celles qui correspondent seulement à des cas très simples.

Sphère de rayon L (volume V)

$$f = 6\pi\eta_0 L \qquad C = 8\pi\eta_0 L^3 \qquad (\eta) = \frac{F}{M} = \frac{2{,}5\,V}{M}$$

Bâtonnet cylindrique d allongement $p > 1$ et de volume V

$$f = 6\pi\eta_0 V^{\frac{1}{3}} \frac{1}{t(p)} \qquad C = 6\eta_0 V \frac{2}{3} \frac{1}{r(p)} \qquad (\eta) = \frac{F}{M} = \frac{V}{M} \frac{2}{3} \Lambda(p)$$

Disques très plat d allongement $p < 1$ et de volume V

$$f = \frac{6\pi}{1\ 612}\eta_0 V^{\frac{1}{3}} \frac{1}{t(p)} \qquad C = 6\eta_0 V \frac{1}{r(p)} \qquad (\eta) = \frac{F}{M} = \frac{V}{M} \Lambda(p)$$

Dans ces équations $t(p)$ $r(p)$ $\Lambda(p)$ sont des fonctions de l al longement calculables par la théorie hydrodynamique des fluides continus.

Il faut bien remarquer qu il n a été possible jusqu ici de pousser la théorie hydrodynamique jusqu aux résultats finaux que pour des particules de forme très simple, l ellipsoïde de révolution représentant le cas le plus compliqué.

Dans le cas des filaments sinueux ou des chaînes en pelote seules des formules empiriques ou semi-empiriques sont utilisables. En général on a $f = kM$ $(\eta) = AM^\beta$ ou k et $\alpha$ d une part A et $\beta$ d autre part, sont des coefficients empiriquement déterminés et valables seulement pour des macromolécules de type chimique déterminé dans un solvant donné.

Si la forme de la particule n est pas connue a priori alors, en principe, les méthodes ci-dessus exposées deviennent inapplicables puisque, encore une fois, les grandeurs morphologiques mesurables dépendent simultanément de la forme et des dimensions des particules.

On peut cependant tenter une estimation de celles-ci de la façon suivante. On admet une forme donnée mettons sphérique et l on calcule les valeurs du rayon L données dans ce cas par les expressions théoriques de f C, F .. Si toutes les valeurs de L coïncident alors on admet que la particule est bien une sphère de rayon L et le problème est résolu.

Si les différentes valeurs de L ne coïncident pas, alors on tente la même opération en supposant que l on a à faire avec un bâtonnet

Dans les cas les plus parfaits (monodispersité rigoureuse, haute précision des mesures) on peut même chercher quelles sont les dimensions de l ellipsoïde de révolution dont les caractères morphologiques (f, C, $\eta$ etc...) correspondent à ceux de la particule réelle.

On arrive ainsi à définir une particule modèle – appelée parfois particule équivalente – dont le comportement en solution est comparable – sinon identique à celui de la particule réelle.

3 *Imperfections et cependant utilité de la méthode*

Mais il faut bien comprendre que la particule équivalente n est qu une image vague de la particule réelle. Il n y a aucune raison – bien au contraire – pour que cette dernière soit un petit bloc de matière compact comme cela est supposé par la théorie. Il suffit, pour s en convaincre, de se rappeler la structure de la myoglobine ou du filament nucléique.

Mais cependant l ensemble des considérations que nous venons d exposer n en sont pas pour autant de simples jeux de l esprit.

D abord il est possible, soit en se livrant à une exploitation approfondie des données expérimentales sur lesquelles nous n avons pas insisté (par exemple dépolarisation de la lumière diffusée, mesures de constantes diélectriques, effet KERR, etc...) soit en comparant les résultats de mesure d expériences de nature physique différente (par exemple en comparant le rayon de giration R déterminé par optique à une dimension L déterminée par hydrodynamique) soit encore en examinant avec soin les structures que les propriétés physicochimiques, ou leur mode de synthèse, imposent aux macromolécules et enfin en se livrant à une discussion étroite de toutes ces données il est possible – répétons nous – d acquérir une image approximative de la particule à l étude.

Ensuite et surtout, même si les grandeurs que permet d atteindre l'expérience peuvent très difficilement conduire à une estimation des formes et des dimensions, il n en reste pas moins qu elles sont en général très sensibles à toute modification de celles-ci. Par conséquent leur emploi est d une nécessité presque impérative lorsqu'il s agit de déceler au cours d une opération, des changements de masse (dégradation) ou de configuration (dénaturation)

Il n y a aucun doute que du perfectionnement des méthodes dont nous venons de décrire les principes dépendent pour une bonne partie, les progrès de nos connaissances sur le comportement des macromolécules biologiques.

Nous nous excusons d'avoir présenté d'une façon aussi superficielle un nouveau domaine de la physicochimie macromoléculaire dont le développement est d'un tel intérêt pour le biologiste, même si encore une fois, les résultats de l'expérience ne sont utilisés que d'une façon semi-empirique.

Nous voudrions seulement rappeler pour en terminer que nous n'avons pas tenté d'insister sur un aspect particulier des développements scientifiques dont nous avons esquissé le schéma : cet aspect, c'est celui de la diversité et de la complexité des disciplines mises en oeuvre.

De la physique quantique jusqu'à la biologie cellulaire, tout doit être mis en oeuvre si l'on veut décrire et expliquer le fait biologique en termes de physico-chimie.

Bien sûr un tel ensemble de notions ne peut-être complètement maîtrisé par un seul cerveau, aussi puissant soit celui-ci.

D'où – là plus qu'ailleurs – la nécessité d'un travail d'equipe mettant en collaboration des hommes dont les domaines d'activité étaient autrefois séparés par d'infranchissables distances. Cette nouvelle forme des structures de la recherche ne peut apparaître, on s'en doute sans de grandes difficultés.

C'est pourtant à la naissance de cette nouvelle époque que nous sommes entrain d'assister.

*Résumé*

Il est important de caractériser les macro-molécules en solution – telles les acides nucléiques ou les protéines – par des caractères dépendant de leur forme ou de la statistique de leurs configurations.

On donne ici un bref exposé des principes sur lesquels reposent les méthodes employées (méthodes hydrodynamiques et méthodes de diffusion du champ électromagnétique) et l'on examine dans quelle mesure leur emploi conduit à la détermination de leurs masses moléculaires ainsi que de celle de leur forme et de leurs dimensions.

*Summary*

It is important to characterize such macromolecules as nucleic acids and proteins in dilute solutions by their morphological parameters.

The principles of the methods of determination of these parameters are briefly renewed:

hydrodynamical methods (determination of viscous coefficients, intrinsic viscosity) or electromagnetic waves diffraction methods (light scattering).

A short discussion shows to what extent molecular weight, shape and dimensions can be derived from a systematic application of these methods.

## Zusammenfassung

Es ist wichtig, Makroglobuline, wie Nukleinsäuren und Proteine, in Lösung nach ihren morphologischen Eigenschaften zu charakterisieren. Die Prinzipien der verwendeten Methoden (hydrodynamische Verfahren und Streuung des elektromagnetischen Feldes) werden kurz besprochen. Es wird untersucht, wie weit damit Molekulargewicht, Form und Größe bestimmt werden können.

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# Therapeutische Fibrinolyse

E. Deutsch

Die thrombolytische Therapie strebt die Wiederauflösung bereits gebildeter arterieller oder venöser Thromben durch Aktivierung proteolytischer, insbesondere fibrinolytischer Fermente an, wobei möglichst keine Nebenerscheinungen entstehen sollten. Dies würde in optimaler Weise dann gelöst sein, wenn es gelänge, die fermentative Aktivität ausschließlich innerhalb des Thrombus zur Wirkung zu bringen, was leider bisher noch nicht möglich ist.

Zur besseren Verständlichkeit seien zwei Definitionen an die Spitze gestellt. Man spricht ganz allgemein von *Fibrinolyse*, wenn Fibrin aufgelöst wird. Ein *fibrinolytischer Zustand* liegt dann vor, wenn das fibrinolytische Ferment (Plasmin) in freier Form im zirkulierenden Blut vorhanden ist. Es greift dann neben Fibrinogen auch andere Gerinnungsfaktoren und verschiedene Eiweißkörper an (3, 30). Außerdem entstehen aus dem Fibrinogen Spaltprodukte, die – je nach ihrer Größe – als Antithrombin oder als Polymerisationsinhibitoren der Gerinnselbildung entgegenwirken. Zerstörung der Gerinnungsfaktoren und Hemmung der Gerinnselbildung verursachen eine schwere Gerinnungsstörung, die sich als hämorrhagische Diathese manifestieren kann.

Von einem *thrombolytischen Zustand* spricht man, wenn Thromben zur Auflösung gebracht werden. Dies kann entweder dadurch erreicht werden, daß fibrinolytisches Ferment von außen auf den Thrombus einwirkt, oder dadurch, daß das im Thrombus enthaltene, an die Fibrinfasern während der Gerinnung adsorbierte Plasminogen aktiviert wird. Infolge des engen Kontaktes zwischen Ferment und Substrat ist ersteres auch dem Einfluß des Antiplasmins entzogen. Es ist verständlich, daß der erstgenannte Mechanismus viel weniger wirksam sein wird als der zweite, was sich auch ganz leicht *in vitro* an einem Gerinnsel zeigen läßt. Außerdem hat die Lyse durch Plasmin von außen her zur Voraussetzung

daß es im Plasma zu einem – an sich gefährlichen – fibrinolytischen Zustand kommt, während die Induktion der Lyse im Thrombus selbst durch einen Aktivator auch ohne Entstehen eines fibrinolytischen *Zustandes im umgebenden Plasma möglich* wäre. *Leider* ist die isolierte Induktion fibrinolytischer Aktivität im Thrombus noch nicht möglich, da der Aktivator nur über die Blutbahn an den Thrombus herangebracht werden kann und man nicht vermeiden kann daß am Transport zum Thrombus eine Aktivierung *des fibrinolytischen Systems im Plasma erfolgt. Es* wird die Aufgabe einer gut geleiteten Therapie sein Zeitdauer und Ausmaß dieses fibrinolytischen Zustandes so kurz und gering als möglich zu halten.

Bevor auf Einzelheiten eingegangen werden soll sollte die Frage diskutiert werden, welche Beweise wir dafür besitzen, daß eine Thrombolyse tatsächlich ausgelöst werden kann

1 Konnte in Tierversuchen bei Hunden und Kaninchen die Lyse von Thromben in peripheren Arterien und Venen (5 10 14 18 32 83 94) in Koronargefäßen (11 13) und nach experimentellen Lungenembolien (15 46) nachgewiesen werden. Völliges Versagen der Anwendung der Streptokinase in Tierversuchen ist auf Speciesunterschiede im fibrinolytischen System zurückzuführen. Bei Pferd, Rind, Schwein und Maus ist die Streptokinase praktisch wirkungslos (1 75) Hier muß menschliches Globulin gleichzeitig gegeben werden. Unter diesen Umständen konnten Martin und Mitarbeiter (67) am Mäuseohr die Lyse von mit elektrischem Strom erzeugten Thromben laufend verfolgen In diesen Versuchen erscheint es allerdings fraglich, ob tatsächlich das fibrinolytische System aktiviert und nicht ein anderer proteolytischer Prozess induziert wurde.

2 Haben Johnson und McCarty (49) schon 1959 gezeigt, daß experimentell erzeugte Thromben in Venen am Unterarm des Menschen durch ein Aktivator Plasminsystem leicht wieder aufgelöst werden können.

3 Spricht die inzwischen erworbene praktisch-klinische Erfahrung auf die später noch näher eingegangen werden wird, im gleichen Sinn.

Im Gegensatz hierzu hat Schmidt (86–89) die Möglichkeit einer Lyse menschlicher Gerinnsel durch Streptokinase durch *in vitro* Versuche in Frage zu stellen versucht *Er fand nämlich, daß* Nativblutgerinnsel, die in Streptokinaselösungen gebracht wurden,

nicht, Gerinnsel aus rekalzifiziertem Zitratblut kaum und Gerinnsel aus rekalzifiziertem Plasma nur teilweise gelöst werden.

Verständlicherweise haben diese Befunde Widerspruch erregt. Wir selbst haben die von KAULLA sche (56) rotierende Standard clotmethode angewendet und Gerinnsel aus Nativblut, rekalzifiziertem Plasma und rekalzifiziertem Vollblut hergestellt. Hierbei fanden wir keinen Unterschied zwischen der Lyse von Gerinnseln aus nativem und rekalzifiziertem Vollblut (Abb. 1) sowie zwischen Gerinnseln aus rekalzifiziertem Vollblut, thrombozytenreichem und thrombozytenarmem Plasma (Abb. 2) Hingegen ergab sich ein deutlicher Unterschied in der Lyse von thrombozytenarmen und -reichen Plasmagerinnseln mit Plasmin (Abb. 3) Zu ähnlichen Ergebnissen kamen andere Autoren mit anderen Methoden (35 42, 51 65 74) Diese Autoren verwendeten teils ebenfalls *in vitro* hergestellte Gerinnsel, zum Teil aber auch *in vivo* entstandene Thromben und konnten zeigen, daß Retraktion der Gerinnsel auf die Lyse hemmend wirkt, der Inhibitorgehalt im verwendeten Blut eine wichtige Rolle spielt, daß aber vor allem die Streptokinase*lösung erneuert oder zumindest in ständiger Bewegung gehalten* werden muß um wirksam zu bleiben. Gerade diese Prämisse fehlt bei den Versuchen von SCHMIDT ist aber *in vivo* gegeben. Es können also die Einwände von SCHMIDT als widerlegt gelten.

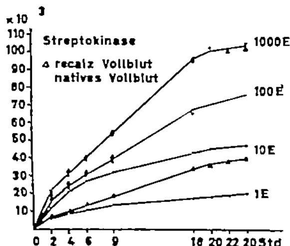



*Abb. 1* Lyse von nativen ( ) und rekalzifizierten (Δ) Vollblutgerinnseln durch Streptokinase im Standardclottest nach von KAULLA. Abszisse: Zeit in Stunden. Ordinate: Länge des gelösten Gerinnselanteiles in mm $10^{-3}$

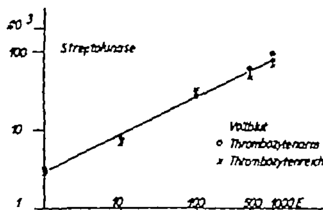



Abb. 2. Lyse von Gerinnseln aus rekalzifiziertem Vollblut, thrombozytenarmem und thrombozytenreichem Plasma durch Streptokinase im Standardclottest nach von KAULLA. Inkubationszeit 4 h. Abszisse: log Streptokinasekonzentration in Einheiten. Ordinate: log der Länge des gelösten Gerinnselanteiles in mm $10^{-3}$

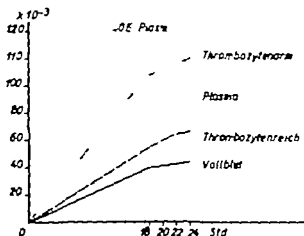



Abb. 3. Lyse von Vollblut, thrombozytenreichen und thrombozytenarmen Plasmagerinnseln durch 50 E Plasmin im Standardclottest nach von KAULLA. Abszisse: Inkubationszeit in Stunden. Ordinate: Länge des gelösten Gerinnselanteiles in mm $10^{-3}$

## *Induktion der Fibrinolyse in vivo*

Zur Auslösung einer fibrinolytischen Aktivität *in vivo* stehen zwei Gruppen von Präparaten zur Verfügung.

1. *Indirekte Fibrinolytica.* Diese greifen selbst nicht in das fibrinolytische System ein, sondern bewirken die Freisetzung eines Aktivators im Organismus.

a) *Pyrogene* Nach der Injektion pyrogener Substanzen kommt es zu einem Absinken der Leukozyten. Hierbei wird wahrscheinlich von den Leukozyten ein Aktivator des fibrinolytischen Systems freigesetzt. Die Wirkung ist kurzdauernd und erschöpft sich bei Wiederholung schnell, die Nebenwirkungen sind unangenehm (21 55)

b) *Adrenalin und Nikotinsäurederivate* setzen bei intravenöser Applikation wahrscheinlich aus den Gefäßendothelzellen einen Aktivator frei. Auch hier ist die Wirkung kurzdauernd und erschöpft sich bei Wiederholung schnell, die Nebenerscheinungen sind groß (23 43)

c) *Sulfonylurea* und *Anabolika* induzieren eine kurzdauernde, *Testosteron*, *Prednison*, *Cortison*, *ACTH* und *Phenformin* (ein Biguanid) eine langdauernde fibrinolytische Aktivität (28)

d) Von Kaulla (57) fand verschiedene *hydrotrope Amine* (z. B. p-Jodbenzoat, 2 4-Dimethylbenzolsulfonat) *in vitro* lytisch aktiv Sie benötigen zur Wirkung menschliches Plasma oder Serum, in dem sie wahrscheinlich einen Aktivator freisetzen.

e) Heparin und insbesondere Heparinoide sind in ihrer fibrinolyseauslösenden Wirkung umstritten (38) Wir selbst konnten nach intravenöser oder subcutaner Applikation von SP 54 in der isolierten Euglobulinfraktion Aktivatoraktivität insbesondere bei Testung an menschlichen Fibrinplatten nachweisen (Abb 4) Der Nachweis der Aktivität im Plasma selbst gelang nur selten. Ähnlich wie in den *in vitro* Versuchen von Olesen (79) besteht auch hier der Verdacht, daß die Erhöhung der Aktivatoraktivität nur durch

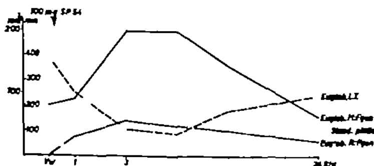



Abb 4 Wirkung von 100 mg SP 54 i.v. auf die Fibrinolyse des Menschen. Normalperson. Standardfibrinplatten nach Astrup mit menschlichem Fibrinogen (——) Rinderfibrinogen (– – —) Euglobulin-Lysezeit (-·-·—) Jeweils wurden Euglobuline getestet. Abszisse Zeit in Stunden nach der Injektion von SP 54; Ordinate gelöste Zone in mm² bzw Euglobulinlysezeit in min.

eine Änderung der Euglobulinfällung in Gegenwart des Polyanions vorgetäuscht wird. SANDRITTER (85) konnte allerdings beim Kaninchen nach SP 54 gleiche Effekte an experimentellen Gerinnungs- und Abscheidungsthromben nachweisen wie mit Streptokinase. Die Übertragbarkeit dieser Beobachtung auf die menschliche Pathologie ist noch nicht eindeutig erwiesen.

Die von diesen indirekten Fibrinolytika induzierten Aktivitäten sind so gering, daß sie kaum ausreichen dürften, um eine Thrombolyse hervorzurufen. Sie haben daher derzeit keine praktische Bedeutung. *Es ist allerdings möglich, daß in Zukunft wirksamere Präparate* entwickelt werden, die für therapeutische Zwecke oder zur Durchführung einer peroralen prophylaktischen Behandlung ausreichen könnten.

2. *Direkte Fibrinolytika.* Diese greifen in das fibrinolytische System direkt ein oder haben selbst enzymatische Aktivität.

a) *Plasmin.* Aus den bereits eingangs geschilderten Gründen ist nicht zu erwarten, daß Plasmin ein besonders geeignetes Thrombolytikum ist. Bei Infusion ausreichender Mengen muß es zur Ausbildung eines fibrinolytischen Zustandes kommen. Da viel Antiplasmin im Blut enthalten ist, wird das Plasmin schnell inaktiviert. Die Annahme von AMBRUS (6), daß das Plasmin aus seiner Bindung an das Antiplasmin wieder freigesetzt wird, sobald es mit Fibrin in Kontakt kommt, konnte in eigenen Untersuchungen nicht bestätigt werden. Es müssen daher so große Mengen des Fermentes verwendet werden, daß es noch aktiv an den Thrombus herankommt. Solche Mengen können kaum aus menschlichem Material hergestellt werden. Daher bestehen bisher nur beschränkte Erfahrungen mit einem Schweineplasminpräparat (7, 8, 62, 92). Betrachtet man diese Ergebnisse kritisch, so fällt auf:

1. daß die Aktivität erst am Ende der Infusion oder gar erst nach der Infusion nachweisbar wird,

2. daß sich gleichzeitig eine Leukopenie ausbildet,

3. daß die nachgewiesene Aktivatoraktivität viel größer als die Plasminaktivität ist, obwohl das Präparat sicherlich frei von Aktivator ist. Diese Ergebnisse legen die Vermutung nahe, daß die gemessenen Effekte gar nicht auf das infundierte Plasmin zurückzuführen sind, sondern daß durch Sekundärreaktionen Aktivator etwa aus den Leukozyten freigesetzt wird.

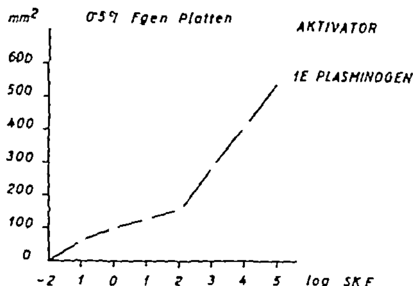
Die meisten in der Literatur als Fibrinolysin bezeichneten Präparate sind in Wirklichkeit Mischungen von Plasminogen und

Streptokinase. Ihre Wirkung ist im wesentlichen auf die enthaltene Streptokinase zurückzuführen (20) Eine Überlegenheit (51) dieser Präparate gegenüber reiner Streptokinase konnte außer bei Tieren, deren fibrinolytisches System durch Streptokinase allein nicht aktiviert werden kann, nicht nachgewiesen werden (25) Die Möglichkeit einer Hepatitisübertragung durch diese Plasminogen enthaltende menschliche Serumfraktion stellt eine zusätzliche Gefahr dar die durch die fragwürdigen Vorteile nicht aufgewogen wird.

b) *Streptokinase* Sie ist das Aktivatorpräparat, mit dem die größten praktischen Erfahrungen bestehen. Sie bildet mit dem Plasminogen eine Verbindung die als Aktivator andere Plasminogenmoleküle zu Plasmin aktiviert. Das Ausmaß der Aktivator und Plasminbildung hängt von der Mengenrelation zwischen Plasminogen und Aktivator ab. Sehr große Mengen Streptokinase unterdrücken die Plasminbildung zugunsten der Aktivatorbildung wie sich leicht *in vitro* zeigen läßt (Abb 5) (59) Sie vermag in den Thrombus selbst hineinzudiffundieren (31 36) und aktiviert dort das am Fibrin adsorbierte Plasminogen das nun ohne mit Inhibitoren in Kontakt zu kommen, ohne Verlust wirksam wird.

Bei der Anwendung von Streptokinase sind einige Schwierigkeiten zu überwinden.

Im Plasma aller Patienten findet sich ein Immunantikörper gegen Streptokinase, der seine Entstehung durchgemachten Streptokokkeninfekten verdankt und dessen Spiegel große individuelle Unterschiede (um etwa das 20-fache) aufweist. Seine Menge muß vor Beginn der Therapie bestimmt werden, da man die Dosis der ersten Injektion so groß wählen muß, daß dieser Antikörper vollkommen aufgebraucht wird. Erst dann kann die Streptokinase wirksam werden (22) Um die zeitraubende Bestimmung der Initialdosis zu umgehen, wurde empfohlen eine Standarddosis von 1,2 Mill. in 30 min zu injizieren, da diese Dosis bei 95% der Bevölkerung zur Neutralisation des Antikörpers ausreicht (98) Dies hat aber den Nachteil, daß man bei einem Großteil der Patienten eine unnötig große Menge eines nicht indifferenten Medikamentes appliziert und daß dadurch eine ohnedies schon nicht billige Therapie noch zusätzlich verteuert wird. Bei Anwendung dieser hohen Dosen muß man auf jeden Fall zur Unterdrückung allergischer Reaktionen Cortisonderivate verabreichen. Wirklich erfolgreich ist nur die kontinuierliche bis zur kompletten Auflösung des Thrombus durchgeführte Therapie. Hierzu verabreicht man nach



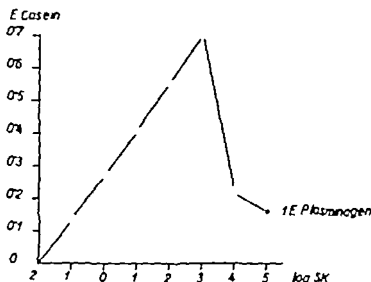




Abb. 5. Mischungen verschiedener Streptokinasekonzentration mit einer Einheit Plasminogen. Obere Hälfte der Abbildung: Aktivatortest. Standardfibrinplatte. Mit zunehmender Streptokinasekonzentration nimmt die Aktivatorkonzentration zu. Abszisse: log Streptokinasekonzentration in Einheiten; Ordinate: gelöste Fläche in mm². Untere Hälfte der Abb.: Plasminbestimmung an Casein. Abszisse: log Streptokinasekonzentration in Einheiten. Ordinate: Jeweils bestimmte Plasminmenge in Caseineinheiten.

Aufbrauch des Plasminogens ⅛ bis ¼ der berechneten Initialdosis pro Stunde (22) oder generell 100 000 Einheiten (98)

Da die Streptokinase nicht direkt in den Thrombus appliziert werden kann, sondern über das Blut an ihn herangebracht werden muß ist die Aktivierung des Plasminogens im strömenden Blut mit allen ihren Folgen nicht zu vermeiden. Um diese so gering als möglich zu halten, sind prinzipiell drei Wege der Applikation denkbar

α) Durch eine besonders vorsichtige Dosierung des Aktivators hat man versucht, die Plasminkonzentration im zirkulierenden Blut so niedrig als möglich zu halten (49) Dies hat dann den Nachteil, daß nur geringe Mengen Aktivator an den Thrombus herangebracht werden. Der größere Nachteil liegt aber darin, daß während der gesamten Dauer der Therapie Plasmin in freier Form im Blut enthalten ist, daß also ein fibrinolytischer Zustand besteht, der zu einer langsamen, aber schließlich mitunter sogar vollkommenen Zerstörung des Fibrinogens und zu allen eingangs geschilderten Folgen führt. Der Vorteil, daß während dieser Therapie kaum neue Thromben gebildet werden können, und daß eventuell doch entstehende Thromben noch Plasminogen enthalten und daher einer weiteren fibrinolytischen Therapie zugänglich sind ist praktisch von geringer Bedeutung

β) Durch eine besonders hohe Dosierung des Aktivators könnte erreicht werden, daß das Plasminogen sehr schnell vollkommen in Plasmin umgewandelt wird (98) Dadurch entsteht zwar vorübergehend eine hohe Plasminaktivität im zirkulierenden Blut, doch dauert diese nur kurz an, da das Plasmin sehr schnell durch das zirkulierende Antiplasmin inaktiviert wird. Da dann weiterhin kein Plasminogen vorhanden ist, bleibt nur mehr Aktivator im zirkulierenden Blut, der dort keinen Schaden anrichten kann. Das vorübergehend abgesunkene Fibrinogen steigt wieder an. Gleichzeitig werden hohe Aktivatormengen an den Thrombus herangebracht. Diese Methode hat den Vorteil, daß ein nur kurzdauernder fibrinolytischer Zustand entsteht, bei dem nur wenig Fibrinogen zerstört und gleichzeitig eine starke thrombolytische Wirkung erzielt wird. Der Nachteil, daß ein eventuell während dieser Zeit entstehender Thrombus plasminogenfrei und daher einer fibrinolytischen Therapie nicht mehr zugänglich ist, muß in Kauf genommen werden, doch wird dies nur selten der Fall sein

γ) Als dritte Möglichkeit ist die gemeinsame Gabe von Streptokinase mit einem Hemmstoff der Aktivierung des Plasminogens

zu nennen. Sowohl Epsilonaminocapronsäure als auch PAMBA verhindern die Wirkung der Streptokinase-Plasminogenverbindung (des Aktivators) nicht aber ihre Bildung. Hierdurch müßte es möglich sein das gesamte Plasminogen in Aktivator umzuwandeln ohne daß sich hierbei ein fibrinolytischer Zustand manifestieren könnte. Die beiden genannten Inhibitoren werden schnell durch die Nieren ausgeschieden, so daß dann der gebildete Aktivator voll zur Wirkung käme. Die kleinen Mengen Plasminogen die kontinuierlich nachgebildet werden, werden durch den infolge der Streptokinase-Infusion ständig vorhandenen Streptokinaseüberschuß sogleich in Aktivator umgewandelt und kommen nicht als Plasmin zur Wirkung. Ein Versuch einer kombinierten Verabreichung von Hemmstoff und Aktivator wurde von uns (24) und von der Arbeitsgruppe von Nilsson (80) gemacht. Allerdings waren die Überlegungen damals noch nicht entsprechend scharf formuliert und die Dosierung beider Komponenten zu vorsichtig als daß der gewünschte Effekt voll erreicht worden wäre (Abb. 6)

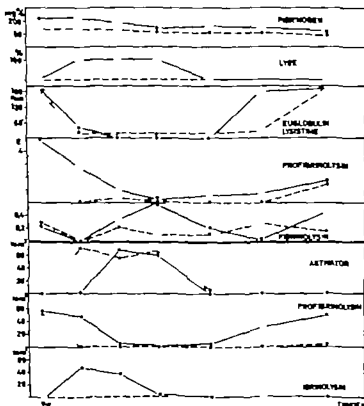
Die lokale Applikation in die Nähe der Thrombose intraarteriell oder intravenös, bietet gegenüber der systemischen Behandlung hinsichtlich der erforderlichen Dosis nur einen geringen, hinsichtlich der Wirkung keinen sicheren Vorteil (14, 15)

*Nebenwirkungen.* Bei den modernen Präparaten sind pyrogene Früh- und Spätreaktionen selten geworden. Allergische Reaktionen sind aber gelegentlich nicht zu vermeiden. Auch folgt auf die Behandlung ein Anstieg des Antistreptokinasetiters der mitunter jahrelang bestehen bleiben und bei einer neuerlichen Behandlung Schwierigkeiten bereiten kann.

c) Die *Urokinase* greift direkt am Plasminogen an und aktiviert es zu Plasmin. Hier handelt es sich um ein humanes Eiweiß, sodaß keine Antikörperbildung induziert wird. Es konnte kein Anstieg des Urokinasehemmkörpers nach der Behandlung beobachtet werden. Auch sind allergische Reaktionen nicht zu erwarten.

Die Empfindlichkeit der einzelnen Patienten weist nur geringe Unterschiede auf (maximal 1 2) (90) sodaß zur Induktion der Thrombolyse eine Standarddosis verwendet werden kann. Es wird anschließend $^1/_2$ – $^2/_3$ der Initialdosis weiter infundiert. Ein weiterer Vorteil besteht darin, daß bereits Dosen eine Thrombolyse hervorrufen sollen, die noch keine Fibrinogenolyse verursachen (50). Allerdings wurde auch bei dieser Therapie eine Abnahme von Fibrinogen und anderen Gerinnungsfaktoren und das Auftreten von

Fibrinspaltprodukten beobachtet. Der breiteren Anwendung der Urokinase steht der außerordentlich hohe Preis entgegen. Die *in vitro* besonders an HAGEMAN Plasma deutliche gerinnungsfördernde Wirkung die wahrscheinlich auf Verunreinigung mit Urothrombo-




—— ohne εACS, ––––– mit εACS.
1 Kolonne Fibrinogen in mg %,
2. Kolonne: Lyse des Plasmagerinnsels in %,
3. Kolonne: Euglobulinlysezeit als Zeichen gleicher Aktivierung,
4 Kolonne: Profibrinolysin, Caseintest, Einheiten,
5. Kolonne Fibrinolysin, Caseintest, Einheiten,
6. Kolonne Aktivator Standardplatten, nach Verdünnung und abdiffundieren der εACS, mm²
7 Kolonne Profibrinolysin, Standardplatten mit Streptokinase,
8. Kolonne Fibrinolysin, erhitzte Fibrinplatten, mm

Abb. 6. Gleichzeitige Infusion von Streptokinase 75 000 E als Erstdosis und dann 100 000 Einheiten pro Stunde und am übernächsten Tag (bei etwas höherer Streptokinaseresistenz) von 225 000 E Anfangsdosis und gleiche Menge pro Stunde gemeinsam mit 5 g εACS pro Stunde bei derselben Versuchsperson.

plastin zurückzuführen ist, scheint *in vivo* keine Rolle zu spielen (50, 70).

d) Andere proteolytische Fermente wie Staphylokinase, Aspergillin O (47, 54) oder Trypsin haben bisher keine praktische Bedeutung erlangt. Wir konnten die orale Wirksamkeit der Asperkinase nicht bestätigen.

*Kontrolle der Therapie*

Die thrombolytische Therapie muß hinsichtlich ihres Einflusses auf das fibrinolytische und das Gerinnungssystem überwacht werden.

1. *Prothrombin* oder *Thrombinzeit* sind schnell und einfach durchführbar und geben einen guten Hinweis auf eine sich eventuell anbahnende Blutungsgefahr. Im Zusammenhang mit der thrombolytischen Therapie werden beide Methoden weitgehend durch die Höhe des Fibrinogenspiegels und die Anwesenheit von Fibrinspaltprodukten beeinflußt.

2. Ergänzt wird dieser Befund durch die *Fibrinogenbestimmung*, wobei am besten die modifizierte Methode nach CLAUSS (17) Verwendung findet (96).

3. Um den Verbrauch des Plasminogens zu erfassen, wird eine *Plasminogenbestimmung* entweder in der von JOHNSON und VERSTRAETE modifizierten CHRISTENSEN-Methode (16, 97) an Fibringerinnseln oder nach NORMAN an Casein (77) als Substrat durchgeführt. Die erstere Methode dürfte etwas empfindlicher sein.

4. *Aktivatorbestimmungen* sind nicht unbedingt erforderlich und technisch etwas schwieriger. Am besten geeignet sind Methoden, die mit $^{131}$I (27) oder Fluoreszenzfarbstoffen (34) markierte, plasminogenangereicherte Gerinnsel verwenden.

5. Es mag auffallen, daß keine Methode zur direkten Erfassung der vorhandenen fibrinolytischen Aktivität für die Routineüberwachung der thrombolytischen Therapie als erforderlich angesehen wird. Dies hat seinen Grund darin, daß alle Methoden langwierig sind oder nur einen beschränkten Aussagewert besitzen. Die *Euglobulinlysezeit* stellt nur einen grob qualitativen Test dar. Bei starker lytischer Aktivität oder Fehlen von Fibrinogen wird sich gar kein Gerinnsel bilden, bei Aufbrauch des Plasminogens wird sich das Gerinnsel nicht lösen, obwohl starke Aktivatoraktivität vorhanden ist. Bei Verwendung des Thrombelastographen wird man am besten jeweils zwei Teste ansetzen, einen mit rekalzifiziertem Blut oder

Plasma zur Erfassung der Gesamtaktivität und einen mit Zugabe von Normalplasma oder plasminogenhaltigem Fibrinogen, um die Aktivatoraktivität nach Aufbrauch des Plasminogens erfassen zu können.

*Indikationen und Ergebnisse*

Die Indikationen zur Behandlung sind noch nicht sicher umschrieben. Die Therapie befindet sich noch im experimentellen Stadium und sollte nur dort durchgeführt werden, wo die personellen Voraussetzungen und die erforderlichen Laboratoriumseinrichtungen vorhanden sind.

Immerhin kann heute bereits gesagt werden, daß *Verschlüsse peripherer Arterien* die bestgesicherte Indikation darstellen. Auf Grund der bisher gewonnenen Erfahrungen sind die Aussichten einer erfolgreichen Thrombolyse bei embolischen Verschlüssen besser als bei autochthonen Thrombosen (41 97 98) Die Therapie muß so lange fortgesetzt werden, bis die Wegsamkeit wiederhergestellt ist, was mehrere Tage dauern kann. Am besten wird ein Team aus Internisten, Gerinnungsfachleuten und Gefäßchirurgen gebildet, die bei einem akut aufgetretenen Verschluß gemeinsam entscheiden, ob chirurgisch oder konservativ vorgegangen werden soll. Dem Chirurgen soll der Vortritt gelassen werden. Erst wenn ein operativer Eingriff auf Grund der Lokalisation oder des Allgemeinzustandes nicht in Frage kommt, soll eine thrombolytische Therapie durchgeführt werden.

Über gut dokumentierte erfolgreiche Behandlung derartiger Fälle (etwa 100) ist wiederholt berichtet worden (19 71 84 91 98 99) Verstraete (98) gibt die Erfolgsquote in der letzten Untersuchungsserie mit 80% an. Auch völlige Wiederherstellung der Passage muß noch nicht zu einem guten klinischen Resultat führen (71) Nach Operationen an Arterien kann eine thrombolytische Therapie vorbeugend oder auch kurativ angezeigt sein ( 3)

Eine weitere Indikation mit der allerdings erst wenig Erfahrungen vorliegen (10 Fälle) ist der Verschluß der *Arteria centralis retinae* (68 91) Wie Böck und Mitarbeiter nachgewiesen haben, ist die Retina noch etwa 10 h lang erholungsfähig so daß eine Behandlung unmittelbar nach Eintritt des Verschlusses eine gewisse Chance bietet, die Sehfähigkeit zu erhalten

Die nächste wichtige aber noch sehr im experimentellen Stadium gelegene Indikation ist die *frische massive Lungenembolie*

Die Therapie hat die Wiedereröffnung der Gefässe und die möglichst schnelle Entlastung des rechten Herzens zum Ziel (ca. 20 Fälle). Es kommt im Rahmen der Therapie zu einem raschen Rückgang der Beschwerden (41–48, 84–91).

Eine thrombolytische Behandlung des *Myokardinfarktes* wäre sehr verlockend. Es kann allerdings kaum erwartet werden, daß ein bereits nekrotisch gewordenes Areale gerettet werden kann. VAN DE LOO (63, 64) hat aber berechnet, daß selbst dann, wenn nur ein wenige Millimeter breiter Randstreifen der Infarktzone durch Lyse der Thromben in den kleinen Kollateralgefäßen oder auch im Hauptgefäß vor der Nekrose bewahrt wird, eine ganz beachtliche Muskelmasse vital bleibt, sodaß schon daraus allein eine theoretische Berechtigung für die Behandlung abgeleitet werden kann. Es sind sicherlich schon insgesamt mehrere 100 Fälle behandelt worden, doch lassen sich die Erfolge nur außerordentlich schwer objektivieren. Es wird von der Ausbildung einer geringeren Zahl transmuraler Infarkte sowie über einen geringeren Anstieg und eine schnellere Normalisierung der Fermente berichtet (12, 26–31, 33, 39, 61, 63, 64, 66, 82, 84–91).

Eine Behandlung cerebraler Gefäßverschlüsse halten wir für sehr gefährlich, obwohl einzelne günstige Erfahrungsberichte vorliegen (9, 52, 93).

Zur Behandlung *venöser Thrombosen* ist der Aufwand in der Regel viel zu groß, sodaß eine venöse Thrombose nur selten eine echte Indikation zur thrombolytischen Therapie darstellen wird. Dennoch ist eine große Anzahl von Patienten mit venösen Thrombosen thrombolytisch behandelt worden, da sich diese Indikation zur ersten Experimentation besonders angeboten hat (4, 22, 29, 34, 41, 52, 53, 60, 76, 84, 91, 99).

Leider sind die meisten Fälle nicht ausreichend phlebographisch dokumentiert und vor allem die von den meisten amerikanischen Autoren vor 1961 erhaltenen Resultate unverwendbar, da die Behandlung mit eindeutig insuffizienten Dosen durchgeführt wurde. Die Ergebnisse stellen lediglich die antiphlogistische Nebenwirkung unter Beweis, die einen guten Behandlungserfolg vortäuscht. Dieser kann schon mit relativ kleinen Dosen erzielt werden. Auf der gleichen Ebene liegen eigene Beobachtungen über einen günstigen Einfluß kleiner Streptokinasedosen auf die postphlebitischen präulcerösen Indurationen der Beine.

Die Befürchtung, daß unter der thrombolytischen Therapie venöser Thromben Lungenembolien auftreten könnten, hat sich praktisch fast nie bewahrheitet.

Die Thrombose der *Vena centralis retinae* (6 Fälle) (41 44 68 72 91) stellt nur eine relative Indikation dar da die Therapie zum Auftreten von Blutungen im gestauten Venenbereich führen und dadurch das Auge gefährden kann.

Bei *Priapismus* sollte die thrombolytische Therapie versucht werden, da die Ergebnisse mit anderen Therapien unbefriedigend sind (58)

Als weitere Indikationen sind das Hyphaema (81 91) und die hyaline Membrankrankheit (4) der Säuglinge zu erwähnen.

Eine sehr interessante, aber noch ganz im experimentellen Stadium gelegene Indikation ist die Behandlung inoperabler Tumoren. Die theoretische Grundlage beruht auf der Beobachtung daß Fibrinfäden als Schiene für das infiltrative Wachstum des Primärtumors und das Annedeln von Karzinomzellen bei der Entstehung von Metastasen dienen, und daß das infiltrative Wachstum von Tumoren um so intensiver ist, eine je geringere proteolytische Aktivität der betreffende Tumor besitzt. Larsen und Mitarbeiter (62) haben einen Rückgang von menschlichen Primärtumoren beobachtet. Clifton hat im Tierexperiment das Angehen von Metastasen durch fibrinolytische Therapie verhindern können (2)

*Kontraindikationen und Nebenwirkungen*

Die Kontraindikationen sind etwa dieselben wie bei der Antikoagulantientherapie. Es wurde bisher über Blutungen aus Ulcera, unvollkommen geheilten Wunden Melaena, zerebrale und subarachnoidale Blutungen, Hämatombildung um arterielle Katheter oder Hämaturie berichtet. Außerdem kann es aus Injektionsstellen bluten.

Durch Gaben entsprechender Dosen von εACS PAMBA oder AMCHA gemeinsam mit Trasylol sowie Ersatz des meist fehlenden Fibrinogens ist es in der Regel leicht möglich, die Blutung zu beherrschen.

Besondere Vorsicht ist wegen der Blutungsgefahr bei der Kombination des fibrinolytischen Agens mit Antikoagulantien (73 91) oder Rheomacrodex geboten. Es ist aber unbedingt erforderlich zur Erhaltung des Therapieerfolges eine Antikoagulantienbehandlung

mit Heparin und Kumarinderivaten unmittelbar an die thrombolytische Behandlung anzuschließen, wobei eine Überkreuzung bei der Einleitung der Kumarintherapie durchaus möglich ist. Allerdings bedarf dieses Vorgehen großer Erfahrung (91, 98).

## *Zusammenfassung*

Zusammenfassend kann gesagt werden, daß die Möglichkeit einer therapeutischen Fibrinolyse als erwiesen gelten kann, wenn auch die Durchführung noch nicht wesentlich über das experimentelle Stadium hinausgekommen ist. Einzelne Indikationen beginnen sich abzugrenzen. Die Suche nach dem optimalen Thrombolytikum ist aber noch lange nicht abgeschlossen.

## *Summary*

There is no doubt that it is possible to perform therapeutic fibrinolysis. This is, however, not yet routine procedure. Some indications are already clear. The best thrombolytic drug is not yet found.

## *Résumé*

En résumé, l'on peut dire que la possibilité d'une fibrinolyse thérapeutique est prouvée quoique son exécution n'ait pas encore dépassé le stade expérimental. Quelques indications commencent par pouvoir être délimitées. La recherche d'un thrombolytique optimal n'est de loin encore terminée.

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# Facteurs locaux du premier temps de l'hémostase

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Aborder le problème de l'hémostase par l étude de ses phases initiales est une entreprise périlleuse car parmi les mécanismes si merveilleusement agencés qui conditionnent l'arrêt des hémorragies, ceux qui en régissent les premières étapes demeurent encore mystérieux.

La découverte du rôle de l adénosine diphosphate (ADP) (7 6, 2) et de sa libération en quantité suffisante par les plaquettes elles-même (21) a permis de préciser le déterminisme de l agrégation, au début réversible, des éléments constitutifs du clou hémostatique, pièce maîtresse de l'hémostase. La mise en évidence d une «atmosphère plasmatique périplaquettaire» (31) son analyse (3 8) révélant l adsorption de tous les facteurs coagulatifs mais l absence d inhibiteurs, l'importance physiologique de la coagulation à la surface même des thrombocytes (28) sont à la base de notre compréhension des stades ultimes de l élaboration du bouchon plaquettaire. De même, la description morphologique de la métamorphose visqueuse (MV) (35) et son étude biochimique (1) expliquent la cohésion et l irréversibilité de cet amas thrombocytaire.

A ces nombreuses informations concernant les stades avancés du phénomène de l hémostase s oppose notre ignorance des facteurs responsables de la fixation des plaquettes à la brèche vasculaire.

L étude de ces phénomènes initiaux se heurte à de multiples difficultés notamment l'extrême rapidité avec laquelle ils se déroulent et les propriétés particulières de la paroi vasculaire. Celle-ci ne peut être remplacée par aucun modèle valable *in vitro* si bien que seules les observations *in vivo* en ce qui concerne ces premiers stades, fournissent des renseignements utiles. Il faut noter aussi, complication supplémentaire, l aspect dynamique de ces événements, arbi-

trairement scindés en étapes successives (fixation agrégation réversible, agrégation irréversible) alors que l étude cinématographique démontre qu ils peuvent être contemporains et se dérouler simultanément en des sites voisins du clou hémostatique.

Les facteurs locaux qui interviennent dans le premier temps de l'hémostase sont multiples. Pour la clarté de l exposé et quoique cette division soit assez artificielle, on peut distinguer deux groupes essentiels les facteurs vasculaires et les facteurs plaquettaires.

Les premiers, indépendants des propriétés particulières des thrombocytes, sont de nature essentiellement *hémodynamique* Ils concernent les variations, consécutives aux traumatisme vasculaire, du calibre du vaisseau, de la vitesse et du débit du courant sanguin à l intérieur de celui-ci.

Les seconds ou *facteurs plaquettaires* sont en relation directe avec les propriétés spécifiques de ces éléments. Ils doivent rendre compte de leur fixation à certaines structures vasculaires ou périvasculaires. Ils conditionnent également leur accolement réciproque et, plus tard, l élaboration d un clou hémostatique efficace parceque parfaitement imperméable au courant sanguin.

Il serait faux de penser que facteurs hémodynamiques et facteurs plaquettaires agissent en parfaite indépendance et nous aurons l occasion de démontrer par quelques exemples que certaines variations de l'hémodynamique locale sont de nature à modifier considérablement la cinétique de ce phénomène si complexe qu est l arrêt des saignements.

## I Les facteurs vasculaires

Alors que de nombreux arguments suggèrent l importance des facteurs hémodynamiques dans l'hémostase, peu de travaux leur ont été consacrés. Cependant dès 1938 Roskam (33) publiait les résultats de ses recherches dans ce domaine, résultats acquis grâce à l'étude des variations du temps de saignement moyen (TSM) valeur statistiquement définie (32) Cet auteur montrait que le TSM au niveau de l oreille du lapin, est prolongé après résection, suivi d une vasodilatation régionale, du ganglion sympathique cervical supérieur Inversement, il note un raccourcissement du TSM pendant la stimulation de l extrémité périphérique de la chaîne sympathique cervicale. Dans certaines conditions de dose et de temps, diverses substances sympathicomimétiques ont le même effet

tandis que les drogues sympathicolytiques prolongent régulièrement les hémorragies.

Ainsi, il était démontré que, sans modifier les facteurs plasmatiques ou plaquettaires, il était possible de perturber le déroulement de l'hémostase.

Devant la complexité des mécanismes régionaux déclenchés par les interventions sur le sympathique, d'autres chercheurs ont abordé le problème du facteur vasculaire par une méthode plus analytique. Leurs observations, au niveau cette fois d'un vaisseau isolé, tendent à préciser l'influence des variations des facteurs hémodynamiques locaux sur l'arrêt des hémorragies.

RIUS et ses coll. (30) ont décrit une technique très élégante qui permet d'enregistrer les modifications du débit sanguin depuis la section du vaisseau jusque la fin du saignement. Ils ont ainsi montré, au cours de recherches préliminaires, que les variations du débit sanguin ne sont pas identiques au niveau des artérioles et des veinules. On peut regretter que leur technique ne rende pas possible la mesure de ce débit avant le traumatisme. On doit craindre aussi, selon les auteurs eux-même, que plusieurs vaisseaux soient parfois sectionnés simultanément. De toute façon, le flot sanguin dont ils estiment l'abondance provient des deux extrémités, proximale et distale, du vaisseau sectionné. Le comportement de ces deux segments pouvant être, ainsi que nous l'avons démontré, très différent, les résultats obtenus sont souvent d'interprétation difficile.

WITTE (39) a évalué les variations du calibre vasculaire de vaisseaux mésentériques assez larges (150 à 300 microns) pendant l'hémorragie. Quoiqu'il n'apporte pas de chiffres précis, il semble démontrer que les vasoconstrictions locales sont indépendantes du système nerveux central ou de l'anesthésie du système orthosympathique, conclusions qui confirment partiellement les résultats que nous avions obtenus.

C'est dans l'espoir de préciser les rapports entre les facteurs hémodynamiques locaux et l'hémostase que nous avons entrepris diverses recherches (12) à l'aide de techniques originales. Celles-ci rendent possible, sans aucune perturbation de la circulation, la mesure du calibre vasculaire, du débit et de la vitesse sanguine avant la section des vaisseaux et, de manière répétée, pendant le saignement jusqu'à son arrêt. Ces observations furent réalisées au niveau des vaisseaux microscopiques du mésentère de lapin, artérioles et veinules d'un diamètre compris entre 20 et 80 microns. Les résultats

essentiels de ces recherches peuvent être résumés de la manière suivante

1 Une *vasoconstriction locale* survient régulièrement après la section des vaisseaux microscopiques. Sa valeur moyenne atteint 31 % du diamètre vasculaire avant la section. La réduction du calibre est plus marquée au niveau des artérioles particulièrement de leurs segments périphériques. Précoce, la sténose post-traumatique se relâche pendant le saignement parallèlement à la constitution du clou hémostatique. Nous avons apporté des arguments en faveur d un mécanisme double de cette vasoconstriction locale d une part la chute de la pression intravasculaire associée à l'élasticité du vaisseau d autre part l'intervention active des éléments musculaires périvasculaires.

Quelle est l influence de la vasoconstriction locale sur l'hémostase?

Pour répondre à cette question, nous avons comparé les temps de saignement individuels de deux groupes de vaisseaux de même nature. Des sténoses post traumatiques discrètes caractérisent le premier groupe, elles sont intenses dans le second. Nous avons également établi des tableaux de corrélation confrontant, pour chaque vaisseau, l importance de la vasoconstriction soit avec la durée de l'hémorragie, soit avec la quantité de sang perdue.

Cette enquête permet d affirmer que, dans nos conditions expérimentales, un seul vaisseau étant lésé, il n existe pas de relation entre l importance de la sténose locale et la qualité de l'hémostase appréciée par les seuls critères valables la durée et l abondance du saignement.

Que de fortes vasoconstrictions ne correspondent pas nécessairement à des saignements courts avait déjà été pressenti par ROSKAM (34) qui avait mis en évidence un allongement du TSM lors du lavage des plaies avec un liquide additionné d éphédrine. Une étude théorique des variations des facteurs hémodynamiques sous l influence des sténoses locales post traumatiques est d ailleurs très instructive à ce point de vue. Elle révèle en effet que des réductions du calibre de 10 à 60 % s accompagnent d une diminution du débit malgré une élévation de la vitesse sanguine. Ces effets sont particulièrement marqués dans les couches périphériques là ou se produit l'accolement des thrombocytes à la paroi traumatisée. Il est logique de penser que l association de ces deux facteurs diminution du nombre de plaquettes disponibles mais augmentation de leur éner

gie cinétique, puisse avoir une influence défavorable sur l arrêt des hémorragies.

2. Le rôle important joué par les facteurs hémodynamiques locaux dans l'hémostase se conçoit plus aisément si on en étudie *les variations pendant l'hémorragie* On constate ainsi que le comportement de ces facteurs est très différent selon la nature du vaisseau sectionné (artériole, veinule, segment proximal, segment distal) Ces différences objectivent le mieux l'influence indéniable de l'hémodynamique dans l arrêt des saignements. Aussi convient-il de les exposer avec quelques détails.

*Au niveau des segments artériels proximaux*, on observe le plus souvent, dès la section vasculaire, une notable augmentation de la vitesse du courant sanguin qui dépasse habituellement 50 mm/sec. (avant la section, vitesse moyenne observée dans les artérioles 6 mm/sec) La sténose post traumatique locale étant ici d'importance modérée, cette augmentation de vitesse va de pair avec une élévation marquée du débit sanguin. Celui-ci diminue ensuite progressivement et souvent irrégulièrement, parallèlement au développement du clou hémostatique. Les hémorragies se prolongent au delà de 4 mm (fig 1)

*Au niveau des segments artériels périphériques* les variations de la vitesse et du débit sanguin pendant l'hémorragie sont totalement différentes. Cette différence s explique par l existence de facteurs dynamiques particuliers à ces segments vasculaires. En effet la

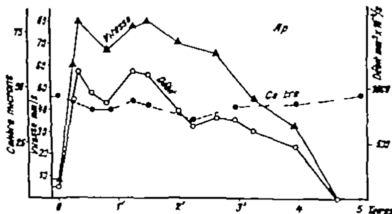



*Fig 1* Variations des facteurs hémodynamiques locaux pendant une hémorragie au niveau d'un segment artériolaire proximal.

section du vaisseau produit ici un renversement du sens du courant sanguin, la brèche vasculaire se trouvant dès lors en aval des résistances capillaires. L'hypotension intravasculaire qui en résulte explique partiellement l'intensité des *sténoses post traumatiques locales*.

A ces deux facteurs chute de la tension et vasoconstriction correspondent sans aucun doute les caractéristiques hémodynamiques *des hémorragies* au niveau *des segments artériolaires périphériques*. On observe en effet, le plus souvent pendant le saignement une chute de la vitesse sanguine et une réduction plus considérable encore du débit. Lorsque l'hémorragie ne se prolonge pas par un suintement persistant, le temps de saignement est généralement court (moins de 2 min) les pertes sanguines faibles (fig 2 et 3)

*Au niveau des segments veineux proximaux ou périphériques* les modifications de la dynamique circulatoire pendant les saignements se situent à mi-chemin entre celles décrites au cours des hémorragies des segments artériels proximaux et périphériques La sténose post traumatique est discrète. L'augmentation de la vitesse et du débit sanguin sont quasi constants mais n'atteignent jamais les valeurs enregistrées au niveau des segments artériolaires proximaux. A ces conditions hémodynamiques correspondent des temps de saignement moyens (environ 2 min) et des pertes sanguines modérées.

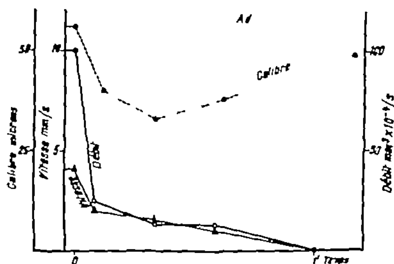



*Fig 2. Variations des facteurs hémodynamiques locaux pendant une hémorragie au niveau d'un segment artériolaire distal.*

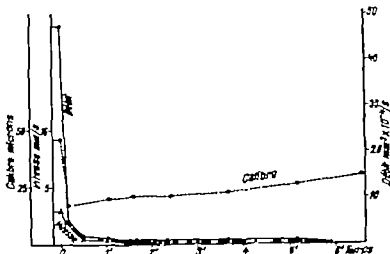



*Fig. 3.* Variations des facteurs hémodynamiques locaux pendant une hémorragie suivie d'un suintement prolongé au niveau d'un segment artériolaire distal.

La moyenne des résultats obtenus au cours d une centaine d ex périences de section réalisées sur les 4 types de segments vasculaires, figure dans le tableau I

Les différences dans la durée des hémorragies ou leur impor tance relèvent donc des variations très diverses de la dynamique circulatoire selon la nature du vaisseau considéré. Il existe enfin pour les veinules et les segments artériolaires proximaux une nette corrélation entre la durée des saignements et la valeur des pertes sanguines. Cette corrélation est beaucoup moins satisfaisante au niveau des segments artériolaires périphériques ce qui s explique par l existence non exceptionnelle de suintements très prolongés après section de ces vaisseaux.

Ces recherches expérimentales et théoriques qui n en sont encore qu à leurs débuts, objectivent, nous semble-t il, l'importance souvent méconnue des facteurs hémodynamiques locaux dans l éla

*Tableau I*

| Vaisseaux | Pertes moyennes (en mm³) | B.T. moyen correspondant |
|---|---|---|
| Artérioles proximales | 6,82 | 4 10'' |
| Artérioles distales | 0,24 | 1 48'' |
| Veinules proximales | 1,32 | 2'06 |
| Veinules distales | 1,29 | 2'19'' |

boration du clou hémostatique. Le développement de ces travaux consacrés à l'aspect quantitatif du phénomène permettra d'évaluer le nombre de thrombocytes disponibles et nécessaires à l'hémostase en fonction des paramètres hémodynamiques caractéristiques du vaisseau sectionné.

## II Les facteurs plaquettaires

Si nos connaissances des mécanismes responsables de la fixation des plaquettes à la brèche vasculaire se sont quelque peu enrichies ces dernières années, il faut cependant reconnaître que nous ignorons toujours le primum movens de ce phénomène.

Un fait très important doit tout d'abord être rappelé c'est l'extrême rapidité avec laquelle les premières plaquettes extravasées adhèrent au vaisseau sectionné. Nous avons montré (14) que, dans 73 % des cas, cette fixation est réalisée dans les 2 premières secondes qui suivent le traumatisme. Dans 80 % des cas elle survient au cours des 3 premières secondes.

Les seuls retards à cette adhésion précoce furent relevés pendant les hémorragies des saignements artériolaires distaux Leur mécanisme s'explique aisément par les variations de l'hémodynamique locale caractéristiques de ces vaisseaux. C'est à leur niveau en effet que l'on observe, au cours du saignement, une nette diminution du débit sanguin, c'est à dire une réduction considérable du nombre de thrombocytes susceptibles d'adhérer précocement à la brèche vasculaire

Maints auteurs se sont interrogés sur la nature de la structure vasculaire ou périvasculaire à laquelle se fixent si rapidement les premières plaquettes extravasées.

Malgré les images histologiques fournies par JORGENSEN et BORCHGREVINK (18 19 20) images qui ne paraissent d'ailleurs pas absolument démonstratives, nous ne croyons pas à l'adhésion des thrombocytes aux *cellules endothéliales* En effet, l'étude de l'ultrastructure du clou hémostatique (22, 17) ne montre que quelques plaquettes isolées en contact probablement fortuit avec l'endothelium. Les observations de BOUNAMEAUX (4) aboutissent aux mêmes conclusions les thrombocytes ne se fixent pas aux cellules endothéliales contenues dans un broyat d'aorte. Faut-il aussi rappeler l'absence d'accolement de ces mêmes éléments à d'autres cellules des organismes supérieurs telles que les cellules amniotiques, à moins

qu elles ne soient chauffées (26) et aussi, dans nos expériences, celles du mésenthelium du mésentère non traumatisé (13)?

Par contre, dès 1955 nous signalions (38) la présence, autour des ébauches de clous hémostatiques, d éléments fibrillaires sur lesquels les plaquettes se fixent avec la même rapidité qu à la brèche vasculaire elle même. En 1959 BOUVAMEAUX (4) constate que les thrombocytes mis en contact de broyat d aorte adhèrent aux fibres conjonctives en suspension dans cette préparation. Nos expériences au niveau des mêmes structures libérées par la dilacération du tissu mésentérique confirment ces faits (13)

Par après, des faits identiques furent rapportés par maints auteurs avec des techniques diverses utilisant le plus souvent des suspensions d extraits aqueux de tissu conjonctif (10 36 40) Enfin, des résultats analogues ont été obtenus par ZUCKER et BORELLI (40) et par nous-même (15) à l aide de collagène purifié.

Il semble donc bien admis à l'heure actuelle que les *structures conjonctives et le collagène* jouissent de la propriété de fixer et d agréger les plaquettes sanguines. Tout porte à croire que ce phénomène joue un rôle essentiel dans les premiers stades de l élaboration du clou thrombocytaire et notamment dans la fixation immédiate des premiers thrombocytes extravasés à la brèche vasculaire. Aussi, dans un rapport consacré au premier temps de l'hémostase con vient il d étudier minutieusement les modalités de cet accolement en s'efforçant d en élucider le mécanisme.

*Fixation des plaquettes aux structures conjonctives*

Il nous est rapidement apparu que l observation *in vivo* (13) de l adhésion des thrombocytes aux fibres conjonctives du mésentère ne permettait pas une étude suffisamment précise et quantitative du phénomène Aussi avons-nous complété ces premiers travaux par des recherches *in vitro* utilisant, contrairement à la plupart des autres chercheurs, un collagène hautement purifié (23)

Deux types d expériences ont été réalisés et leurs résultats confrontés. Les uns ont pour but l observation microscopique de la fixa tion des thrombocytes à des fibres de collagène en suspension entre lame et lamelle. Ils permettent d étudier les transformations mor phologiques subies par les plaquettes dans ces conditions. Les autres s efforcent, par le recours à des techniques photométriques, de pré ciser la cinétique de ce phénomène et son inhibition par certains

agents. Des échantillons prélevés dans la cuve du photomètre et examinés au microscope permettent le contrôle des éventuelles altérations plaquettaires

Toutes les observations microscopiques sont réalisées au microscope de phase Les propriétés les quantités et la stabilité du collagène purifié que nous utilisons sont détaillées dans un travail ultérieur (16) Nous pensons utile d exposer les principaux résultats de ces travaux et de les confronter avec ceux obtenus par d autres dans le même domaine.

*A Observations microscopiques entre lame et lamelle*

Le collagène purifié, en solution isotonique dépourvue de calcium est précipité sous forme de fibrilles. Le plasma riche en plaquettes (PRP) additionné d anticoagulant, est introduit par capillarité au contact des éléments fibrillaires.

Les plaquettes se fixent *immédiatement* aux fibrilles de collagène. La rapidité du phénomène rappelle évidemment celle de l accolement des mêmes éléments aux fibres conjonctives du mésentère dilacéré et surtout la précocité de l adhésion des premiers thrombocytes extravasés à la brèche vasculaire.

L accolement des plaquettes aux fibres de collagène résiste au *lavage et au tapotage. Quelques plaquettes restent isolées et de* même certaines fibres de collagène ne fixent aucun thrombocyte. La fixation au niveau des autres fibres est d importance variable.

Dans les minutes qui suivent leur fixation immédiate aux fibres de collagène, les plaquettes subissent d importantes transformations morphologiques. Celles-ci débutent après 2 à 4 minutes et consistent tout d abord en un étalement des plaquettes isolées le long des fibres de collagène. Les thrombocytes accolés entrent ainsi en contact les uns avec les autres et dès lors fusionnent rapidement. Après 5 à 10 minutes, les limites individuelles des éléments ne sont plus visibles en microscopie de phase. L homogénéisation de l amas plaquettaire se complète au cours du 1^er^ quart d'heure Parfois il semble que des granules soient libérées de cet agglutinat qui englue progressivement les fibres de collagène. Assez souvent et avec une fréquence qui augmente avec la minceur de la préparation, on voit apparaître au contour des amas plaquettaires des éléments sphériques vésiculaires de faible réfringence Enfin une contraction de l amas plaquettaire parait souvent se produire tardivement.

*Tableau II*

| Agents inhibiteurs (+) | Fixation précoce | Homogénéisation |
|---|---|---|
| Plaquettes normales | +++ | +++ |
| Lavage des plaquettes | +++ | + |
| Réserpine | +++ | + |
| Prométhazine | + | — |
| Antiserotonine (Ro3–1442) | + | — |
| Fluorure de sodium | ++ | — |
| Chlorure mercurique | ++ | + |
| Citrate de soude (2 % final) | ++ | ++ |
| EDTA (0,1 % final) | +++ | ++ |
| EDTA (0,5 % final) | ++ | + |
| Héparine (1 % final) | ++++ | ++++ |
| AMP (0,16 % final) | +++ | ++ |
| Adénosine (0,04 % final) | +++ | ++ |
| Plaquettes de Glanzmann | ++ | + |
| Plaquettes d'Hémophilie A | +++ | +++ |
| Plaquettes de von Willebrand | +++ | +++ |
| Acide hyaluronique | +++ | +++ |
| Chondroïtine sulfate | ++++ | ++++ |

(+) Le nombre de +++ schématise, de manière semi quantitative l'importance du phénomène. Les concentrations non précisées sont consignées dans un travail antérieur (15).

Les plaquettes isolées dans la préparation, sans contact avec les fibres de collagène, ne subissent pas, dans nos conditions expérimentales, ces transformations morphologiques. Tout au plus et de manière exceptionnelle, constate-t-on la présence de quelques amas faits de l'accolement de 2 à 4 plaquettes avec granulations encore bien individualisées.

Aucun filament de fibrine n'est visible dans la préparation, même après de longs délais.

Il est logique de distinguer dans l'adhésion des thrombocytes au collagène au moins deux étapes: la fixation immédiate et les altérations morphologiques secondaires. En effet, ces phénomènes ne sont pas simultanés et il est possible à l'aide de certains inhibiteurs d'entraver électivement le développement de l'un ou l'autre de ces stades. Les résultats de ces essais d'inhibition spécifique sont mentionnés dans le tableau II.

Quelques détails méritent d'être soulignés:

a) l'action inhibitrice de l'EDTA varie avec sa concentration. L'anticoagulant n'entrave l'homogénéisation des amas fibres-plaquettes qu'à forte concentration (fig. 4 a et b).



*Fig. 4.* a) Aspect dense et homogène d'un amas mixte plaquettes-fibres de collagène dans des conditions normales. b) Adhésion parfaite mais absence d'homogénéisation en présence d'EDTA. Obj. 40× Largeur champ 0,26 mm.



*Fig 5.* Aspect normal d'amas mixtes plaquettes-fibres en présence d'AMP Obj. 40×
Largeur champ. 0,26 mm.

b) Dans nos conditions expérimentales, l AMP et l'adénosine ne sont que des inhibiteurs imparfaits. Ces substances retardent seulement le déroulement du phénomène étudié mais ne l'entravent pas complètement (fig 5)

c) L application de collagénase au niveau des agrégats pla quettaires, même homogénéisés, provoque la disparition des éléments fibrillaires avec dissociation partielle des amas et libération de thrombocytes isolés.

d) Enfin, d autres particules telles que les corpuscules d encre de Chine se fixent, comme les thrombocytes tant aux fibres conjonc tives du tissu mésentérique dilacéré qu aux fibrilles de collagène *in vitro*

*B Observations photométriques*

Nous utilisons du PRP dont le taux de plaquettes est ajusté à 500.000 éléments par mm³ par dilution avec du plasma pauvre en plaquettes (PPP) provenant des mêmes animaux. Un mélange de PRP et de LP (NaCl 0 9 ) est introduit dans la cuvette thermosta tisée d un photomètre, soumis à une agitation électromagnétique

standardisée et incubé dans ces conditions pendant au moins 5 min avant l addition de collagène. Pendant cette incubation, la turbidité de la suspension ne présente pas de variations. Après le contact avec un agent agrégant, la transmission optique, mesurée toutes les 15 s, augmente.

Les volumes et les concentrations des différents milieux utilisés sont précisés dans un travail plus détaillé (16)

Additionnée en petite quantité à du PRP dilué, la solution de collagène provoque une clarification de la suspension. Cet effet est lié à l apparition d amas plaquettaires denses et homogènes, ne contenant pas d éléments fibrillaires de taille décelable au microscope de phase. L effet agrégant ne se manifeste qu après un délai compris entre 1 et 3 min dans les conditions normales (courbe I de la *fig 6*) Une fois déclenché, ce phénomène suit un décours parallèle à celui de l agrégation induite par l'ADP (courbe II) Aucune clarification de la suspension ne s observe dans les expériences témoins où soit le collagène est remplacé par du L.P (courbe III) soit le PRP par du PPP (courbe IV)

L action du collagène soluble est fonction de la température qui modifie les deux paramètres de la courbe d une part la période de latence, d autre part le maximum de la transmission optique. Les meilleurs résultats sont obtenus aux environs de 32 C (fig 7)

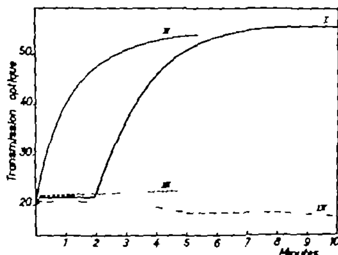



*Fig 6.* Etude photométrique de l'action d'une solution de collagène purifié (courbe I) ou d'une solution d'ADP (courbe II) sur le PRP du L.P sur le PRP (courbe III) d'une solution de collagène purifié sur le PPP (courbe IV)

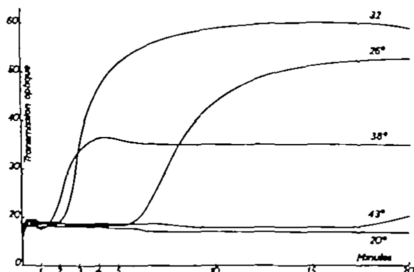



Fig 7 Action, à diverses températures, d'une solution de collagène purifié sur le PRP

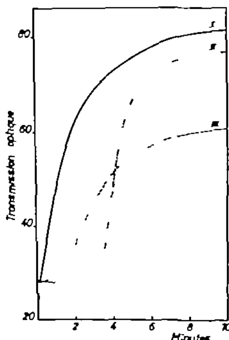



Fig 8 Courbe I Action d'une suspension de fibres de collagène sur le PRP – Courbe II Action d'une solution de collagène sur le PRP – Courbe III Action sur le PRP du surnageant obtenu après centrifugation de la suspension d'amas mixtes fibres de collagène-plaquettes.

Cette influence de la température paraît liée à la polymérisation des molécules de collagène sous forme de fibrilles ou de protofibrilles. Plaide dans ce sens la comparaison des effets obtenus par des quantités égales de collagène soit en solution soit transformé en fibrilles.

Dans le dernier cas en effet l'effet agrégant se manifeste d'emblée, sans aucun délai (fig 8 courbe I collagène en fibrilles, courbe II collagène en solution) On peut en conclure, semble-t il, que les molécules isolées de collagène sont sans effet et que l'accolement des thrombocytes ne survient qu'en présence d'éléments fibrillaires de dimensions d'ailleurs sub-microscopiques.

La période de latence après addition de collagène soluble correspondrait à la polymérisation des molécules protéiques sous l'influence du chauffage.

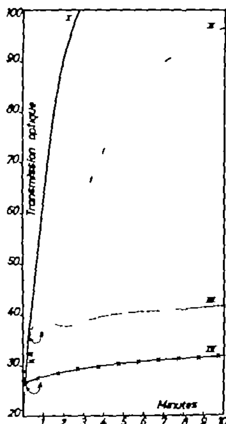



Fig 9 Courbe I Action d'une suspension de fibres de collagène sur le PRP Courbe II Idem, en présence d'AMP Courbe III Idem en présence d'EDTA. Courbe IV Idem en présence de prométhazine.

La courbe III montre l effet du surnageant obtenu par centri fugation à froid de la suspension des amas fibres-plaquettes 10 mm. après l addition de collagène en fibrilles. Cet effet est vraisemblablement lié à la présence d ADP libéré, ainsi que Hovig l a démontré (11) par le contact des thrombocytes avec le collagène.

Le recours à divers inhibiteurs permet de reconnaître dans nos observations photométriques, les différentes étapes de l'accolement des plaquettes aux fibres de collagène La courbe I de la figure 9 montre le déroulement normal de ce phénomène déclenché par l addition d une suspension de fibres à du PRP En présence de prométhazine (courbe IV) on n observe quasi aucune variation de la transmission optique. Les plaquettes prélevées dans ces conditions sont d ailleurs parfaitement dispersées avec quelques petits amas exceptionnels faits de 2–3 éléments accolés. Elles paraissent en outre, altérations déjà bien connues, arrondies et denses (fig 10)

L action inhibitrice de l EDTA en forte concentration (courbe III) et de l AMP (courbe II) préalablement ajoutés au PRP se manifeste plus tardivement dans le développement du phénomène (à partir du point B de la fig 9) Avec l'anticoagulant à forte dose, la clarification de la suspension demeure discrète. L examen mi

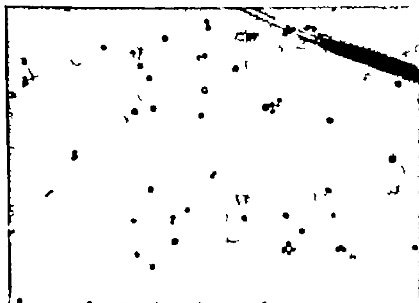


*Fig 10* Examen microscopique d'un échantillon prélevé dans la cuv photométrique correspondant à la courbe IV de la Fig 9 Obj. 40 x Largeur champ 0,26 mm.

croscopique des échantillons prélevés dans ces conditions fournit des informations très intéressantes confirmant les expériences entre lame et lamelle. On constate en effet la présence de thrombocytes *assez nombreux, fixés aux fibrilles mais sans aucune tendance à l'éta*



11

12

*Fig. 11.* Examen microscopique d'un échantillon prélevé dans la cuve photométrique correspondant la courbe III de la Fig. 9. Obj. 40× Largeur champ 0,26 mm.

*Fig. 12.* Examen microscopique d'un echantillon prélevé dans la cuve photométrique correspondant la courbe II de la Fig. 9. Obj. 40× Largeur champ 0,26 mm.

lement et à la formation de volumineux amas mixtes homogénéisés (fig 11)

La présence d AMP (courbe II de la fig 9) retarde seulement la réaction sans l entraver complètement. Les agrégats plaquettaires présentent une structure analogue à celle élaborée dans des conditions normales (fig 12)

Enfin la technique photométrique permet aussi d objectiver l action agrégante du collagène sur d autres particules telles que les corpuscules d encre de Chine (fig 13) Il semble cependant que la cinétique de ce phénomène diffère de celle de l accolement des thrombocytes notamment par l absence de période de latence que le collagène soit utilisé sous forme soluble ou sous forme de suspension de fibrilles.

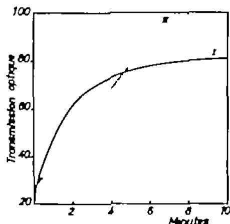



*Fig 13.* Courbe I Action d'une suspension de fibres de collagène sur le PRP Courbe II Action d'une suspension de fibres de collagène sur une suspension d'encre de Chine.

### C *Interprétation des résultats*

Si les résultats obtenus par différents auteurs (10 36, 40) concordent en général avec les nôtres, il convient cependant de noter quelques divergences. Elles peuvent être attribuées soit aux propriétés physico-chimiques du collagène purifié comparées à celles des extraits bruts de tissu conjonctif soit à l emploi de techniques souvent très différentes.

Ainsi Zucker et Borrelli (40) notamment ont décrit la réversibilité des agrégats plaquettaires formés au contact d extraits bruts de tissu conjonctif. Dans nos conditions expérimentales, les amas

thrombocytaires bien homogénéisés ne se dissocient pas au cours d observations prolongées pendant plus d une heure, sauf cependant sous l influence de la collagénase. Par ailleurs, nous n observons pas, contrairement à d autres (37 11) une inhibition totale de l accolement par l addition préalable d AMP ou d adénosine. La trypsine, enfin, qui, selon SPAET et ZUCKER (37) entrave le phénomène, est sans action dans nos conditions expérimentales.

Notre connaissance des mécanismes responsables du 1$^{er}$ stade de l *hémostase est enrichie par les résultats acquis récemment par* divers chercheurs dans ce domaine. Signalons par exemple la libération par les plaquettes après leur contact avec les structures conjonctives, de 5 HT d ADP (37 11) la présence très précoce de ce nucléotide au niveau des plaies vasculaires (24) les propriétés de divers agents provoquant l agrégation des thrombocytes et de leurs *inhibiteurs spécifiques* (27) *Des* renseignements très utiles ont également été acquis grâce à l examen de l ultrastructure des clous hémostatiques (22 17) ou des agrégats induits par la thrombine, ou l ADP (9 29) Mentionnons enfin la description du rôle des lysosomes plaquettaires (5) et les recherches concernant la phagocytose par thrombocytes de diverses particules ou agrégats protéiques (25)

C'est à l aide de ces diverses informations que l on peut tenter d élucider le déterminisme des étapes successives de l adhésion des thrombocytes au collagène.

1 La fixation des plaquettes aux fibres de collagène est *immédiate* Dès lors et à moins que l on n admette la présence locale de traces de thrombine élaborée à la suite d un processus physiologique de coagulation continue, il est peu probable que des phénomènes coagulatifs interviennent à ce stade. L absence de toute inhibition par l héparine à forte dose plaide dans le même sens.

En fait, dans nos conditions expérimentales, peu d agents s avèrent capables d entraver cette adhésion précoce. On retiendra cependant l action manifeste de la prométhazine et de certains autres antagonistes de la sérotonine Que cette amine joue un rôle important dans cette étape initiale ne semble cependant pas pouvoir être retenu car cela supposerait sa libération extrêmement rapide dès le contact avec le collagène Il nous paraît plus logique de penser que les indéniables altérations plaquettaires induites par ces drogues (plaquettes denses et arrondies) modifient la répartition de leurs charges électriques superficielles, charges qui interviendraient de manière active dans leur accolement sans délai aux structures fibrillaires.

On se rappelera aussi, à propos de la prométhazine, que les antihistaminiques stabilisent la membrane des lysosomes entravant les processus de phagocytose. Ceux-ci ont été incriminés par certains (35) dans l adhésion des thrombocytes au collagène. La valeur de cette hypothèse demande à être précisée par de nouvelles recherches mais dès à présent, on notera que l EDTA entrave la phagocytose mais non la fixation précoce des plaquettes aux fibres de collagène.

Divers arguments détaillés dans un travail antérieur (15) rendent également peu vraisemblable l intervention, à ce stade de l ADP Les expériences après addition d AMP et d adénosine con firment cette opinion.

En conclusion, on doit admettre que le mécanisme de la fixa tion immédiate des thrombocytes demeure encore mystérieux. Ce phénomène, éventuellement de nature électrostatique nécessite une structuration particulière du collagène et la présence non pas de molécules isolées de cette protéine mais leur agencement en fibrilles ou proto-fibrilles.

2 *L homogénéisation des amas plaquettes-fibres* rappelle les altéra tions morphologiques décrites sous le nom de «métamorphose visqueuse» Mentionnons les similitudes même aspect en microscopie de phase et au microscope électronique, dégranulation des éléments et contractions des amas. Rappelons aussi la «release reaction» présente dans les deux cas avec libération de 5 HT d ADP et du PF3 De nombreux inhibiteurs identiques agissent sur les deux phé nomènes les lavages répétés des thrombocytes, la réserpine des antagonistes de la sérotonine, la prométhazine, le citrate de soude et l EDTA, l un et l autre à forte concentration. Enfin, les plaquet tes de thrombasthénie de GLANZMANN ne sont pas agrégées par la thrombine et ne subissent pas d'homogénéisation après leur fixation sur le collagène. Malgré quelques divergences (action du fluorure de soude de la collagénase et de l'héparine – peut-être neutralisée localement par le PF4 –) on ne peut manquer d être frappé par ces analogies, d autant plus que HOVIG (9) nous a appris que de peti tes doses de thrombine provoquent l agrégation des plaquettes sans précipitation de fibrine.

Aussi, après les travaux de JOHNSON (17) qui révèlent la pré sence très précoce de fibrine, c est à dire de thrombine dans l'ébau che du clou hémostatique, sommes-nous enclin à penser que les trans formations morphologiques subies par les thrombocytes dans les minutes qui suivent leur fixation au collagène, sont provoquées par

des traces de thrombine. L'enzyme pourrait être élaboré en petite quantité par des processus coagulatifs se déroulant à la surface des plaquettes ou représenter un des chaînons d'une éventuelle coagulation physiologique continue.

Enfin, quelque soit le déterminisme de la «release reaction» subie par les thrombocytes au contact du collagène, l'ADP libéré intervient certainement pour faciliter l'accolement consécutif de nouvelles plaquettes extravasées. Mais si le rôle de ce nucléotide est important, il ne nous paraît pas de nature à élucider totalement le mécanisme si complexe des stades initiaux de la constitution du clou hémostatique.


## *Résumé*

Les facteurs locaux du 1er temps de l'hémostase sont essentiellement de nature hémodynamique et plaquettaire.

Les premiers, analysés grâce à des techniques originales, sont représentés par les variations du calibre vasculaire, du débit et de la vitesse du courant sanguin après la section du vaisseau. Ces variations dépendent de la nature du segment vasculaire observé. Elles conditionnent de manière importante le déroulement de l'hémostase et sont en rapport direct avec la durée et l'abondance des saignements.

Les propriétés particulières des thrombocytes doivent expliquer leur adhésion très rapide à la brèche vasculaire. Cette adhésion se produit non pas au niveau des cellules endothéliales mais bien au contact de structures conjonctives périvasculaires. Le primum movens de ce phénomène demeure encore mystérieux. Après leur fixation immédiate aux éléments conjonctifs, les plaquettes subissent des transformations morphologiques qui rappellent celles décrites sous la dénomination de métamorphose visqueuse



## *Summary*

Local factors in the first phase of haemostasis are essentially those of haemodynamic nature and those of platelet origin.

The first, which has been studied by new methods, has been shown to consist of changes in the vascular lumen, cardiac output and rate of blood flow after section of the vessel. These changes depend on the nature of the segment of vessel under study They have a marked effect on the process of haemostasis and are therefore in direct relationship with the duration and amount of haemorrhage

Platelet factors must account for their very rapid adhesion to the defect in the vessel wall. This is determined not in the endothelial cells but by contact with perivascular connective structures. The prime mover in this phenomenon remains mystery *After immediate fixation to these connectiv elements, the platelets undergo morphological changes resembling those described as viscous metamorphosis*



## *Zusammenfassung*

Die lokalen Faktoren der ersten Phase der Blutstillung sind vorwiegend hämodynamischer und thrombocytärer Natur Bei den ersteren, die mit den Originalmethoden untersucht wurden, handelte es sich um Veränderungen des Gefäßkalibers, des Vo-

maßes und der Geschwindigkeit der Blutströmung. Diese Variationen hängen von der Natur des untersuchten Gefäßsegmentes ab. Sie bestimmen wesentlich den Ablauf der Hämostase und stehen in direkter Beziehung zur Dauer und zum Umfang der Blutungen. Die besonderen Eigenschaften der Thrombocyten erklären ihre sehr rasche Adhäsion an der Gefäßfläche. Diese Adhäsion spielt sich nicht auf dem Niveau der Endothelien ab, sondern vielmehr im Kontakt mit perivaskulären Bindegewebsstrukturen. Die eigentliche Ursache dieses Phänomens bleibt vorerst ungeklärt. Nach ihrer unmittelbaren Fixation an Bindegewebselemente zeigen die Plättchen morphologische Veränderungen, die an diejenigen der visköSen Metamorphose erinnern.

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# Importance of Coagulation besides Haemostasis

F Koller

It has become traditional that blood clotting is discussed almost exclusively in connection with haemostasis and thrombosis. But if the rapid formation of a solid blood clot is often life saving in case of injury coagulation has to fulfill other important functions, which have been rather neglected in the past.

The end product of coagulation being insoluble fibrin, we shall discuss first the clotting process as a whole and particularly the rôle of fibrin in certain biological reactions, and in a second part we shall review briefly the multiple functions of isolated clotting factors or of "intermediate clotting products.

Coagulation of fibrinogen is of particular importance in *inflammation*. In the early stages of this reaction, when the capillaries dilate and the rate of blood flow diminishes, an exudation of fluid becomes apparent. Owing to the increased capillary permeability fibrinogen and other clotting factors escape with this exudate into the intercellular spaces where coagulation takes place. It is logical to assume that this fibrin formation is produced by the extrinsic clotting system, in other words by tissue thromboplastin—at least in the beginning

What is the significance of this fibrin-network in the inflammatory reaction? It appears to be an important factor (not the only one) in the *fixation* of bacteria and other injuring agents, in keeping them localized in the lesion and in preventing their propagation. Menkin has shown conclusively that the spreading of bacteria is markedly reduced by an inflammatory reaction of the peritoneum for instance. Not only the fibrin deposits in the tissue spaces, but also in the lymphatics (the so-called "lymphatic thrombus ) appear to be important in this respect. It is probable that this localizing effect is not only due to the mechanical protection of the surroundings by a fibrin layer (the latter is not so dense as to prevent the passage of bacteria) but also to the powerful *adsorption* of bacteria and other particles onto the fibrin network.

There is however one serious objection to the above mentioned assumption the often repeated statement that in patients with *afibrinogenemia* (who are unable to form any fibrin not even a loose one!) delayed wound healing apparently does not occur

In Switzerland, several cases of practically complete afibrinogenemia have been observed in recent years by GUGLER. He found that healing of small injuries is very much delayed and produces big and ugly scars, whereas large wounds usually heal quite normally It was not difficult to explain this apparent paradox large wounds always need extensive transfusions which supply enough fibrinogen for normal wound healing whereas small injuries are treated without transfusions, so that the lack of fibrinogen becomes apparent. Normal wound healing in afibrinogenemia seems thus to be one of the many erroneous statements which are taken over from one author to the other

GUGLER observed moreover in a afibrinogenemic patient with peritonitis a very inadequate inflammatory reaction (although a voluminous serous exudate had accumulated the peritoneum was neither hyperemic nor thickened) which became normal only after transfusion. These observations do certainly not contradict but rather confirm the importance of fibrin in inflammation and wound healing

The rôle of coagulation which we just mentioned is certainly beneficial to the individual (unless there is an excess of fibrin deposit) There are, however other functions of the clotting process which are definitely detrimental to the organism. It has been demonstrated recently that *metastases of malignant tumors* get implanted in various organs only if the tumor cells are surrounded by clotted blood. In experimental studies, CLIFFTON WOOD and others have shown that the vast majority of tumor emboli—single cells or group of cells—perish in the blood stream. Only those survive which become attached to the vascular endothelium of capillaries, small arteries or veins and get embedded in a delicate meshwork of fibrin Several of these emboli may aggregate and form a *tumor thrombus* It is highly probable that the thromboplastic properties of carcinoma cells (being often in excess of that of normal cells) are responsible for this clotting phenomenon. The tumor thrombus has possibly a nutritive value for the malignant cells.

As a direct consequence of these observations, attemps have been made to reduce the implantation of tumor emboli by anti

coagulants as well as artificial fibrinolysis. In experimental studies, heparin, dicumarol and fibrinolytic agents all produced a decreased metastasis rate. One clinical trial from Denmark with artificial fibrinolysis maintained during weeks or even months arrived at a similar conclusion (LARSEN, MOGENSEN, AMRIS and STORM).

These micro thrombi (usually in the capillaries) are sometimes accompanied by venous thrombosis; thrombophlebitis migrans sive recidivans is often seen in metastasizing tumors of the pancreas, the lungs etc.

In some of these cases even a *generalised intra-vascular clotting* is observed which produces a widespread consumption of clotting factors and as a consequence a severe bleeding syndrome.

There is another condition, where fibrin formation is of importance, I mean the so-called *fibrinoid degeneration in collagen diseases*. KLINGE was the first to recognise characteristic histologic lesions in connective tissue as the common denominator of this group of diseases. He also proposed the designation "fibrinoid degeneration". In fact, we are dealing with a deposit of "fibrinoid" in the ground substance of connective tissue, and the main controversy has been whether this fibrinoid is an autochthonous alteration of this ground substance or if it represents extravasated plasma components. By various methods it has been shown conclusively that fibrinoid is—for the most part at least—derived from plasma proteins.

But the question arises what plasma proteins produce this fibrinoid? Is it really—as the name suggests—fibrin, in other words clotted fibrinogen? Fluorescent antibody technique has demonstrated that in the majority of collagen diseases fibrinoid is not derived from fibrinogen but represents gammaglobulin. Only in the renal lesions of scleroderma—as in malignant hypertension—real fibrin can be demonstrated in the wall of arterioles, (not in connective tissue). The same holds true of renal cortical necrosis, where fibrinogen clots in the lumen of the small vessels as well as in their wall. Renal cortical necrosis is found in the experimental SHWARTZMAN-SANARELLI phenomenon and also in clinical conditions, in certain complications of pregnancy, in shock, in other words in the already mentioned generalised intra vascular coagulation.

In summary fibrinoid consists mostly of γ-globulins, in rare instances however as in certain lesions of scleroderma it is composed of real fibrin and therefore produced by a clotting process.

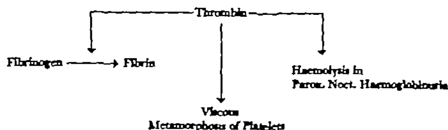



*Fig 1*

We have studied up to now coagulation as a whole, in other words, the rôle of fibrin formation. In concluding I should like to draw your attention to a few *special functions of certain isolated clotting products!*

Thrombin e.g. plays an important rôle not only in the conversion of fibrinogen to fibrin but also in *viscous metamorphosis of platelets*. Furthermore, it enhances *haemolysis* in paroxysmal nocturnal haemoglobinuria; this increased haemolysis may promote again generalised intravascular coagulation. The mechanism of these actions of thrombin is still obscure.

In the very beginning of the clotting process HAGEMAN Factor (Factor XII) appears to fulfill several functions. Not only it sets in motion, after activation by surface contact, the whole clotting process, leading by a series of successive enzymatic reactions to fibrin, but at the same time it appears to induce other reactions

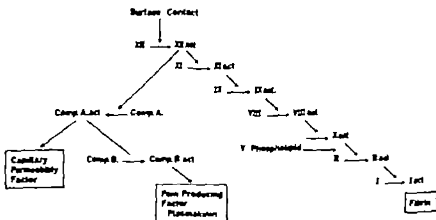



*Fig 2*

with plasma components, producing the so-called permeability factor of Miles as well as the pain producing substance of Keele or *plasmakinin*. These are factors which are of greatest importance in the inflammatory reaction.

Fig 2 which summarizes the concept of MARGOLIS, MAC FARLANE a. o is still tentative, but the connection between blood clotting and other mechanisms involved in inflammation appears to be firmly established. These examples demonstrate that blood clotting is by no means limited to haemostasis and thrombosis but that besides a wide field is opened for coagulation research.


## *Summary*

Coagulation plays an important rôle in inflammation (particularly fibrinous inflammation) and wound healing furthermore in the implantation of tumor metastases, in certain collagen diseases (scleroderma) with "fibrinoid" deposits etc. Possible therapeutic implications are discussed.

The multiple functions (besides coagulation) of certain activated clotting factors (HAGEMAN Factor thrombin) are outlined.

## *Zusammenfassung*

Die Gerinnung spielt eine wichtige Rolle bei der Entzündung (insbesondere der fibrinoiden Entzündung) und bei der Wundheilung, ferner bei der Implantation von Tumormetastasen, bei gewissen Kollagenkrankheiten (Sklerodermie) mit "fibrinoiden Ablagerungen etc. Die möglichen therapeutischen Folgerungen werden diskutiert. Die mehrfachen Funktionen (neben der Gerinnung) gewisser Faktoren (Hageman-Faktor Thrombin) werden besprochen.

## *Résumé*

La coagulation joue un rôle important dans l'inflammation (particulièrement dans l'inflammation fibrineuse) dans la cicatrisation des plaies, ainsi que dans l'implantation de metastases tumoreuses, dans certaines maladies du collagène (sclerodermie) comprenant des dépôts "fibrinoïdes etc. Les conséquences thérapeutiques possibles sont discutées.

Les multiples fonctions (à part celle de la coagulation) de certains facteurs (facteur de Hageman activé thrombine) sont esquissées.


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# The Nature of the Defects in Hemorrhagic Disorders

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Hemostasis in a restricted sence is usually defined as the arrest of bleeding following a break in the vascular system. In a broader sence it would include also the normal maintenance of vascular integrity, preventing the leakage of blood. The mechanism of hemostasis involves the interdependence of a number of factors such as blood platelets, blood coagulation, vascular contraction, hemodynamic factors etc. These various mechanisms are closely integrated and spontaneous bleeding often requires the involvement of more than one elementary factor.

For the purpose of analysing the principal defects in the various hemorrhagic disorders I shall firstly discuss primary defects in the local hemostatic mechanism as triggered by injury of normal vessels. Secondly, I shall discuss the vascular defects.

## *Primary Defects in the Mechanism of Arresting Bleeding*

By direct observation of living blood vessels the first observable hemostatic reaction following injury of the vessel wall consists of adherence of platelets to the intima at the edge of the severed vessel, particularly to collagen fibres. Secondly, new platelets adhere in succession to those already arrested, forming loose aggregates. These are permeable to the blood flow and are steadily formed, disrupted and rebuilt. The platelet morphology remains normal (Fig. 1). This is the stage of reversible platelet aggregation. The third main reaction occurs after some minutes and includes structural platelet changes with loss of granula and mitochondria. The platelet clumps or plugs become more confluent and better coherent with loss of platelet individuality as seen in the light microscope but with preserved membranes, as observed by electron microscopy (Fig. 2). This



*Fig. 1* The stage of reversible platelet aggregation. Normal platelet morphology. ADP induced aggregate ( 50,000; Hovig, 1962)

stage leads to impermeability of the platelet plug and provisional arrest of bleeding. As a fourth reaction a perimetric fibrin formation presumably produced by the coagulation of plasma is observed.

It is generally agreed that the formation of hemostatic platelet plugs provide for the mechanical arrest of bleeding following injury of arterioles and venules. It has recently been demonstrated by histological studies of primary and secondary hemostasis that platelet plugs also quite often take part in the arrest of capillary bleedings (1). Bleedings from capillaries are otherwise arrested by endothelial adhesion and coagulation. The following scheme may therefore, serve as a basic framework for our discussion on the various defects of these mechanisms which may occur clinically (Fig. 3). The formation of the hemostatic platelet plug is favoured by other mechanisms such as the pressure of tissue fluids, the plasma surface tension and the immediate and late vasoconstriction, but these factors are of secondary importance.



*Fig 2* The stage of viscous metamorphosis characterized by degranulation Thrombin-induced aggregate ( 20,000 Hovig, 1962)

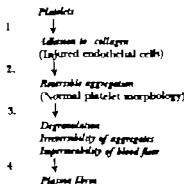



*Fig* 3. The main stages in the hemostatic mechanism.

## *Defective Adhesion of Platelets to Collagen Fibres*

This first adhesion takes place within 1–3 s after injury. Adhesion to damaged endothelial cells probably also takes place but this is less important for hemostasis. Adhesion to collagen is independent on calcium and can be studied separately by suspension of washed collagen particles in EDTA-plasma. The reaction does not seem to be dependent of any of the known plasma factors and we have found virtually normal adhesion in patients with von Willebrand's disease and in thrombasthenia. Hemorrhagic disorders caused by defective adhesion is listed in Fig. 4.

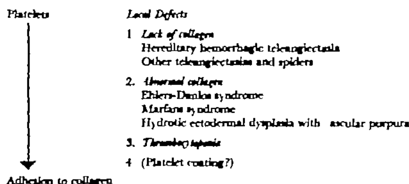



*Fig. 4.* Causes of defective adhesion of blood platelets to collagen fibres.

## *Defective Collagen*

The presence of normal collagen is indispensable for normal adhesion. A local lack of collagen fibres as a cause of hemorrhagic disorders is seen by injury or spontaneous rupture of the vessel wall in hereditary teleangiectasias (Rendu-Osler-Weber's disease). The wall of the vessels at the local dilatations has been reduced to the endothelial layer only and the small wound surface which is produced by rupture is practically devoid of collagen fibres and consequently will be inefficient for initiating platelet plug formation. The dysplasia of the ground substance and vessels in Osler's disease is most pronounced on the venous side of the capillaries. A similar situation is also seen in teleangiectasias and spiders associated with other diseases.

The problem of an abnormal structure of the collagen as a cause of ineffectiveness in attracting platelets and triggering the liber

ation of adenosine diphosphate from the platelets, has not yet been analysed. Abnormal collagen is a likely explanation for the hemorrhagic tendency in Ehlers-Danlos syndrome, Marfans syndrome and Hydrotic ectodermal dysplasia. Ehlers-Danlos syndrome is caused by a mesenchymal dysplasia which also involves the blood vessels. The fibres of the connective tissue show fragmentation and degeneration. Marfans syndrome also shows abnormalities of the collagen and connective tissue. Of particular interest is the fact that these patients excrete large amounts of hydroxyproline in the urine. Hydroxyproline is a rather specific constituent of collagen tissue.

The number of adhering platelets will easily be insufficient in thrombocytopenia. Whether adhesion is defective of platelets coated with abnormal protein such as in dysproteinemias has not yet been investigated.

### *Deficient Platelet Aggregation*

The reversible platelet aggregation has been intensively studied in recent years. According to the present state of knowledge normal aggregation depends on the presence of the five factors listed in Fig 5. It seems now to be generally agreed that HELLEM's (2) red cell factor (factor R) which was shown by GAARDER *et al* (3) to be adenosine diphosphate, is the physiological trigger of this reaction. Being a normal intracellular substance, ADP will always be available at the site of cellular injury where it is also formed by a break down of adenosine triphosphate by an effect of tissue ATP ase. More important however is the release of ADP from platelets by contact with collagen as demonstrated by HOVIG (4) and others. Additional ADP is probably generated from platelet adenosine triphosphate by an effect of platelet ATP ase, including thrombostenin which has ATP-ase activity. A supplementation of ADP seems to originate from the red blood cells from which it is released probably by their contact with the wound surface.

A reduced supplementation of ADP from red cells might explain the old clinical observation first reported by DUKE in 1910 (5) that patients with severe anemia generally have a prolonged bleeding time. HELLEM *et al.* (6) showed that by transfusion of red cells to such patients the platelet adhesion-aggregation tendency increased and the bleeding time decreased similarly to the rising hematocrit value and they both became normal at normal hematocrit.

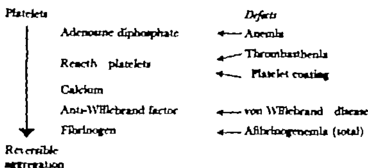



*Fig 5.* Various defects causing disturbance of reversible platelet aggregation.

A deficient release of ADP from the platelets by contact with collagen as a cause of hemorrhagic disease has not been demonstrated but might be an additional factor in precipitating the prolongation of the bleeding time in dysproteinemias (multiple myeloma amyloidosis, macroglobulinemia Waldenstrom dysproteinemias associated with malignant disease) The main defect in these disorders however is the non reactivity of the platelets on exposure to ADP Reduced release of ADP has been observed in thrombocytopathia and in myeloid leucemia (7)

There are several hemorrhagic disorders which are caused by defective platelet aggregation. An obvious cause is thrombocytopenia which however also reduces capillary resistance and which I shall comment upon later

Reversible aggregation requires viable platelets with normal glycolysis. They become non reactive to ADP by prolonged storage and by substances which block glycolysis such as iodo-acetate. The lack of reactivity is most pronounced in congenital thrombasthenia in which platelets are completely refractory even to high concentrations of ADP The diagnosis of this condition is very simple therefore because it is the only known disorder in which the addition of adenosine diphosphate in excess of 0.2 $\mu g/ml$ to citrated platelet rich plasma do not cause visible aggregation of blood platelets (Fig 6) This simple test which takes a few seconds only is pathognomonic for thrombasthenia and should be performed in all cases with normal platelet count and prolonged primary bleeding time The basic metabolic disturbance in the thrombasthenia platelets is in the glycolytic system (8) Normal glycolysis therefore, seems to be necessary for maintaining an appropriate surface of the blood platelets for the attachment of ADP and consequent aggregation.

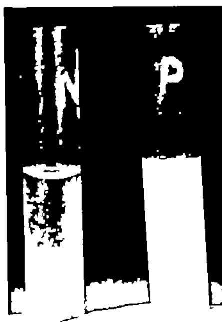



*Fig* 6. The addition of ADP in excess of 0.2 μg/ml gives strong aggregation in normal plasma (N) but no aggregation in plasma from patients with thrombasthenia (P)

In the dysproteinemias already mentioned, with coating of the platelets with abnormal proteins, we have found a very marked reduction in their ability to aggregate on exposure to ADP By HELLEM's technique values of 1–10 % were found (9) This is evidently the main cause of the prolonged bleeding time in these disorders.

Coating of platelets seems also to occur after multiple blood transfusions and is then probably caused by platelet isoantibodies. This situation is particularly important to recognize after transfusions in hemophilia The primary bleeding time, which is normal in uncomplicated hemophilia becomes prolonged and renewed transfusions tend to increase the bleeding tendency Cortison therapy shortens the bleeding time to normal in these patients but has no effect on the prolonged bleeding time in dysproteinemias. Dextrane (Macrodex) is also adsorbed on to the platelets and massive infusions may cause platelet coating and prolongation of the bleeding time.

Another important disease with normal platelet number and prolonged primary bleeding time is von Willebrand's disease. This

is probably the most interesting disease within this group It is associated with a deficiency of the antihemophilic A factor (factor VIII) but this deficiency is not so severe as to cause, by itself any significant disturbance of hemostasis. Renewed interest in the pathogenetic mechanism of this disease was evoked by the investigations of NILSSON BLOMBÄCK and BLOMBÄCK (10) in Sweden who demonstrated that infusion of large amounts of normal plateletfree plasma or of plasma fraction I–0 or even of hemophilic plasma, at least partly corrected the prolonged bleeding time. It has been amply confirmed that the basic defect in this disorder is the lack of a plasmatic hemostatic or anti bleeding factor which I have called for convenience the anti Willebrand factor A characteristic *in vitro* finding is that addition of ADP in excess of 0 2 μg/ml to citrated platelet rich VON WILLEBRAND's plasma causes largely normal aggregation but the addition of 0 05 μg/ml gives minimal or no adhesion aggregation as tested by HELLEM's method in contrast to the marked aggregation produced in normal plasma and in coagulopathias (11) (Fig 7)

It may also be mentioned that an abnormally high activity of anti Willebrand factor presumably causing increased aggregation tendency might to be an important factor in determining the increased tendency to thrombosis which is associated with atherosclerotic disease diabetes, and the postoperative state etc.

| | Adhesive platelets % | | |
|---|---|---|---|
| ADP μg/ml | 0.10 | 0 05 | 0.025 |
| Normal (average) | 51 | 25 | 12 |
| v. Willebrand's disease (5) | 40 | 0 | 0 |
| Hemophilia A (5) | 53 | 35 | 17 |
| Hemophilia B (3) | 51 | 29 | 13 |
| Anticoagulant-treated pat. (5) | | | |
| TT 5 hematuria | 57 | 30 | 17 |

*Fig 7* The addition of ADP to [illegible] final concentration of 0 05 μg/ml or less to plasma from patients with von Willebrand's disease produces no platelet adhesion-aggregation as tested by HELLEM's method in contrast to the marked effect produced in normal plasma and in coagulopathias ([illegible])

The last factor which is required for reversible aggregation is plasma fibrinogen. Deficient platelet aggregation and prolonged bleeding time is seen only at extremely low concentrations of fibrinogen approaching zero. The defect produced by lack of fibrinogen is complex because the following reactions of irreversibility of the platelet aggregates and the formation of plasma fibrin are also defective.

The aggregation of platelets by ADP is inhibited by AMP and ATP as shown by BORN (13) and also by a number of other substances (14). The prolonged primary bleeding time in uremic patients is probably caused by defective aggregation (15).

*Defective Viscous Metamorphosis and Irreversibility of the Aggregates*

The relation between blood coagulation and hemostasis which until lately remained in the realm of medical mystery was largely clarified by the discovery of the platelet surface clotting system and its function in hemostasis. Viscous metamorphosis and irreversible aggregation is triggered by thrombin, which we believe is provided by clotting factors adsorbed on to the platelet surface and not by the coagulation of plasma (16). This conclusion was based on the finding that transfusion of normal platelets without plasma restored the prolonged primary and secondary bleeding time to normal in various coagulopathies (17). Exposure of platelets to thrombin is followed by a series of reactions of which the degranulation and release of platelet substances and the production of irreversibility and impermeability of the aggregates, providing for arrest of the bleeding, are the most important ones (Fig. 9).

If thrombin formation on the platelet surface is defective, the hemostatic platelet plug will remain permeable to the blood flow and the bleeding time becomes prolonged. Both the extrinsic and the intrinsic platelet surface clotting system are triggered and take part in hemostasis by a primary injury to the vessel wall. Tissue thromboplastin is released, triggering the extrinsic system and the intrinsic system is triggered by a mechanism which has not yet been definitely clarified. The normal or nearly normal primary bleeding time in the presence of a defective intrinsic clotting system, such as in severe hemophilia, and also by a defective extrinsic system, such

as in complete factor VII deficiency shows that only one of the platelet surface clotting systems is required for provisional primary hemostasis. This explains why the primary bleeding time is normal in coagulation disturbances which affect only one of the clotting systems. If both systems are depressed however such as by over dosage with oral anticoagulants, the primary bleeding time becomes prolonged. The main cause of bleedings in anticoagulant therapy is either a combined deficiency of factor VII of the extrinsic system and of factor IX of the intrinsic system or and that is more frequent, a severe depression of factor X (Stuart Prower factor) which takes part in both clotting systems (18)

Congenital deficiencies of factor X, prothrombin or factor V are extremely seldom of such severity as to give a prolongation of the primary bleeding time

A characteristic finding in disturbances of the intrinsic clotting system is a normal primary and a prolonged secondary bleeding time. This difference between the two tests is caused by the fact that gentle removing of the crust 20–24 h after the primary bleeding time test has been done will produce minimal new cellular damage and consequently thromboplastin, which is an intracellular substance will not be released in efficient amounts for triggering the extrinsic clotting system Hemostasis therefore, will be dependent only on the intrinsic clotting system If this system is deficient such as in hemophilia, renewed bleedings provoked at the site of a primary injury tends to be prolonged and difficult to arrest. The local defect in hemophilia therefore, is first and foremost at the site of secondary bleeding and consists in a lack of viscous metamorphosis and

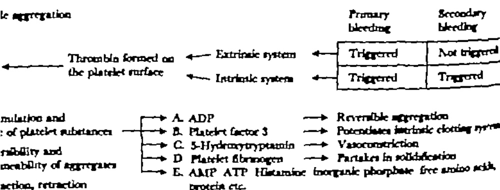



*Fig 8.* Various effects on blood platelets by thrombin.

irreversibility of the platelet aggregates. The situation is also similar in other severe disturbances of the intrinsic clotting system, except in deficiencies of the HAGEMAN factor (factor XII) and of PTA (factor XI). The explanation of this paradox seems to be that the intrinsic clotting system in vivo is also triggered in the absence of the HAGEMAN factor and probably also of PTA, presumably because of direct activation of the anti-hemophilic B factor (factor IX).

The significance of fibrinogen for viscous metamorphosis and irreversibility of the platelet plug has not yet been definitely settled. Fibrinogen is specifically adsorbed on the platelet surface and could likely be the substrate for thrombin. The observation that papain and reptilase which also convert fibrinogen into fibrin, induces viscous metamorphosis speaks in favour of this concept. The theory has been questioned however because viscous metamorphosis has been reported to take place normally in plasma from patients with congenital afibrinogenemia. In a case of complete congenital afibrinogenemia, SOKAL (19) found that platelet aggregates were formed during coagulation of native plasma and degranulation occurred, but the platelets started to segregate after 2–4 h. This might indicate that platelet fibrinogen is not necessary for degranulation or viscous metamorphosis, but is required for gluing the platelets irreversibly together. The glue would then be fibrin. Platelets have priority for residual plasma fibrinogen because of specific adsorption and the lack of irreversibility is seen only in complete afibrinogenemia.

Bleeding in coagulation disturbances are usually provoked by trauma although this may be slight or unnoticed. Whether true spontaneous bleedings occur is disputed. This question is related to the problem of whether normal coagulation is required for the maintenance of normal vascular integrity.

## *Primary Vascular Defects*

Vascular anomalies as a cause of defective platelet hemostasis have already been discussed. Bleedings caused by other vascular disturbances are usually spontaneous and most often result from increased fragility or permeability giving the clinical picture of vascular purpura. The occurrence of such bleedings depends on the status of vascular resistance on one side and the stress and strain which the vessels are exposed to on the other side. A decrease in vascular resistance below certain critical levels may give rise to

petecchial bleedings either spontaneously or only by increasing the strain such as by the positive or negative pressure tests. When results of these tests are evaluated one should keep in mind that the blood vessels are readily influenced by a number of variable physiological factors such as the blood pressure the extravascular pressure, the state of the autonomic nervous system and hormonal factors.

The nature of the endothelial defect which cause extravasation of blood is poorly understood. Bleedings in scurvy are usually considered an example of increased vascular permeability and it is believed that red cells in this disorder escape through the intercellular cement substance, the synthesis of which is assumed to be dependent on ascorbic acid. Recent investigations by FLOREY *et al.* (20) have shown however that the cement substance probably does not exist. This problem is closely related to the physiological mechanism which maintains normal endothelial resistance, a complicated question which has not yet been solved. One theory maintains that a continuous physiological intravascular coagulation takes place with deposition of fibrin on the vascular endothelium and that this deposition is important for protecting the integrity of the capillaries. A delicate "hemostatic balance has been postulated between a fibrin depositing hemostatic system and a removing fibrinolytic system (21) Certain clinical observations are consistent with this theory such as the occasional occurrence of purpura in congenital clotting defects, during anticoagulant therapy and in fibrinolytic states. Purpura is an exception rather than the rule however in these conditions and the evidence in favour of this theory is far from conclusive

Another theory is based on the fact that platelets are essential for the integrity of the small vessels. Purpuric hemorrhages and increased capillary permeability and fragility are frequent findings in platelet disorders. The mechanism of the endothelial supporting function of platelets however is unknown. It has been speculated that platelets or platelet fragments are lining the endothelial wall as an endo-endothelial film (22, 23) protecting the intercellular spaces and continuously preventing or repairing small vascular lesions resulting from normal "wear and tear JOHNSON *et al.* (24) have studied electron micrographs of ultrathin sections of capillaries in the dermis from patients with thrombocytopenia after platelet transfusions and observed that platelets probably become incorporated into the endothelial cytoplasm. It is difficult however to visualize the importance of this process for capillary integrity

The structural changes which occur in purpura caused by an antigen antibody interaction has recently been analysed by MOVAT and FERNANDO (25) By the use of electronic microscope they found that gaps appeared in the venules at junctions between two or more endothelial cells. Both the antigen antibody precipitates, the red cells and platelets escaped through these gaps. Except for the separation between adjacent endothelial elements there was no alteration of these cells. As stasis developed, red cells accumulated in venules and capillaries, but extravasation occurred only from venules. No alterations were observed in small arteries or capillaries. Similar vascular gaps have been observed also by MAJNO and PALLADE (26) in the endothelium of venules, affected by histamine or serotonin. Purpuric bleedings have however been observed also from the capillary loop (27) from the terminal arteriole (28 29) and from the venous side of the capillary It is possible therefore, that the actual site of extravasation of red cells may vary depending both on experimental technique and on the primary cause of vascular damage

Capillary resistance is affected by a large number of factors local factors (area, temperature of the skin) emotional and physical stress, nutritional deficiencies and pharmacological factors such as adrenalin, adrenocrom, histamine antagonists, cortison etc. The effect of various stress factors have been analysed particularly by JACQUES *et al.* (30) in experimental animals. They have found that a breakdown of the clotting mechanism does not produce spontaneous bleeding except when superimposed by certain types of stress and strain on the vessels. These findings are in agreement with clinical experiences in congenital clotting deficiencies in which bleedings regularly are precipitated by an additional trauma or vascular strain. Of particular interest is the recent finding of SALMON (31) that a prerequisite for purpura in thrombocytopenia is the presence of increased fibrinolytic activity which he assumes has a direct effect on the vascular endothelium. Thrombocytopenia without increased fibrinolysis was not associated with clinical purpura. It is a clinical experience that there is no good correlation between the tendency to spontaneous bleedings and the degree of thrombocytopenia, nor is there any good correlation between the severity of a congenital clotting disturbance and the bleeding tendency Hemophilic patients with a stable coagulation defect show great variations in bleeding tendency from time to time. They have good periods with no bleedings and bad periods with frequent bleedings. The

problem of vascular integrity and its relation not only to coagulation and blood platelets, but also to other factors, hormonal, emotional nutritional etc., therefore deserves further study

Vascular contraction is also believed to be of importance for hemostasis. Injury of a small artery or arteriole is quickly followed by a temporary and strictly local vasoconstriction probably mediated by an axonic reflex. The consecutive retardation of blood flow might conceivably favour the formation of the hemostatic platelet plug Greater importance has been ascribed to the delayed or more extensive and persistent vasoconstriction which is caused by the release of platelet substances during viscous metamorphosis. This contraction has been ascribed to an effect of serotonin (5-hydroxytryptamin) The role of serotonin in hemostasis seems doubtful however since elimination of serotonin from the blood platelets by treatment with reserpin do not result in any disturbance of the hemostatic process.

In conclusion it may be said that research in recent years have largely clarified the mechanism of hemostasis and enabled us to *localize the main defect in this mechanism caused by the various dis*orders of coagulation and platelet function, but the exact nature of and the role played by disturbances of vascular integrity in the various hemorrhagic disorders is far from clarified.

*Summary*

The nature of the defect in hemorrhagic diseases caused by disorders of blood coagulation and platelet function has been largely clarified in recent years through an improving knowledge of the hemostatic mechanism. Hemorrhagic disease can be caused by defective or abnormal collagen associated with defective platelet adhesion (hereditary hemorrhagic telangiectasia, Ehlers-Danlos syndrome Marfans syndrome etc.) by deficient platelet aggregation (thrombasthenia, platelet coating" von Willebrand's disease) and by defective viscous metamorphosis and consolidation of the platelet aggregates (certain coagulation anomalies) The nature of the primary defect in vascular hemorrhagic disorders is poorly understood.

*Zusammenfassung*

Die Natur des Defektes bei hämorrhagischen Diathesen infolge Störungen von Gerinnung und Plättchenfunktion wurde in den vergangenen Jahren durch Verbesserung der Kenntnisse über den Hämostasemechanismus weitgehend geklärt. Eine hämorrhagische Diathese kann bedingt sein durch fehlendes oder abnormes Kollagen mit einem Defekt der Plättchenadhäsion (hereditäre hämorrhagische Teleangiektasie Ehlers-Danlos-Syndrom und Marfan-Syndrom usw.) durch mangelhafte Plättchen-

aggregation (Thrombasthenie, platelet coating von Willebrandsche Krankheit) und durch unvollständige visköse Metamorphose und Konsolidierung der Plättchenaggregate (gewisse Gerinnungsanomalien). Die Natur der primären Störung bei vaskulären hämorrhagischen Diathesen ist noch weitgehend ungeklärt.


## Résumé

La nature des défectuosités rencontrées dans les maladies hémorragiques causées par des troubles de la coagulation sanguine et de la fonction des thrombocytes été largement clarifiée au cours de ces dernières années grâce à l'amélioration des connaissances sur le mécanisme hémostatique. Une maladie hémorragique peut être causée par du collagène défectueux ou anormal associé à une adhésion défectueuse des thrombocytes (télangiectasie hémorragique héréditaire, syndrome de Ehlers-Danlos, syndrome de Marfan etc.) par une aggrégation déficientes des thrombocytes (thrombasthénie, platelet coatings maladie de Willebrand) et par une métamorphose visqueuse ainsi qu une consolidation défectueuses des aggrégats de thrombocytes (certaines anomalies de la coagulation) La nature du trouble primaire dans les désordres hémorragiques vasculaires est encore mal comprise.


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# Chromosomes and Leukaemia

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It is not surprising that the remarkable rate of progress achieved in chromosome studies in the leukaemias from 1959 to 1962 has not been maintained more recently. It seems likely that renewed progress must await either the development of new methods of study or the adoption, with or without modification, of methods already developed in other fields of research but as yet unexploited in the study of leukaemia. The present time is, then, an appropriate one to assess the accomplishments of the past six years, to attempt to define the problems for future study and perhaps to try to foresee the direction, if not the outcome, of further investigations.

The possibility that the presently available methods of chromosome study in leukaemia may still yield important new results cannot be dismissed. Nevertheless, it is a striking fact that the knowledge already gained has been acquired step-wise as new techniques have become available. The earliest results which revealed aneuploidy and morphological abnormalities of chromosomes in some cases of acute leukaemia (Ford, Jacobs and Lajtha, 1958; Baikie, Court Brown, Jacobs and Milne, 1959) were obtained by combining short-term culture and colchicine treatment of bone marrow with the recently developed methods for making human metaphase preparations. The introduction of the method for culturing peripheral blood leucocytes (Moorhead, Nowell, Mellman, Battips and Hungerford, 1960) led to a higher standard of chromosome preparations. This improvement made possible the detection of the Philadelphia chromosome (Nowell and Hungerford, 1960; Baikie, Court Brown, Buckton, Harnden, Jacobs and Tough, 1960) which had been overlooked in preparations made from cultured marrow. Comparison of the results of simultaneous observations on bone marrow and blood cultures pointed to important differences between the

two tissues (Baikie, Jacobs, McBride and Tough 1961) These comparisons explained the fallacies which may arise from the occurrence of differential growth and selection in cultures of mixed cell populations. Attention had first been drawn to this possibility by Ford and Mole (1959) About the same time it was shown (Mackinney Stohlman and Brecher, 1962) that phytohaemagglutinin stimulated normal peripheral blood lymphocytes to divide in culture The problem of fallacious results was largely solved by the introduction of methods which made possible the study of human bone marrow without preliminary culture (Sandberg Koepf Crosswhite and Hauschka, 1960 Tjio and Whang 1962) Studies of bone marrow by these methods have been especially valuable in the exploitation of the discovery of the Philadelphia chromosome and in the confirmation of its specificity for cells of bone marrow origin in chronic granulocytic leukaemia.

All these technical innovations have concerned the selection of cells for study and their preliminary preparation. Actual chromosome preparations are still examined at or near the limit of resolution of the light microscope Although the Philadelphia chromosome is a normal chromosome which has lost as much as 40 of its substance (Rudkin Hungerford and Nowell, 1964) yet it could have escaped detection for longer than it did. Even large deletions or translocations involving the larger chromosomes would remain undetected by current methods of examination. Some improvement in magnification or visualization may be obtained by electron microscopy (Barnicot and Huxley 1961 Osgood Jenkins, Brooks and Lawson 1964) or by interference microscopy (Lonowell, 1961) both methods having major attendant disadvantages which limit their usefulness. Phase contrast provides, at best, only doubtful improvement on bright field microscopy Progress in the future may depend on the combination of morphological observations with methods dependent on the chemistry of the chromosomes. Thus, Rudkin *et al* (1964) have measured the DNA content of individual chromosomes in chronic granulocytic leukaemia and radioactive thymidine labelling has been used by Muldal, Lajtha and Gilbert (1965) in the same disease More remote prospects for progress include the combination of morphological observations and newer methods of DNA analysis.

Consideration of the present technical imperfections should remind us that we do not know whether acute leukaemia and chronic

lymphocytic leukaemia may not have their own specific chromosomal abnormalities, analogous to the Philadelphia chromosome of chronic granulocytic leukaemia. A second point which should not be overlooked is that we have no way of knowing whether the cells in chronic lymphocytic leukaemia whose chromosomes have been examined and pronounced indistinguishable from normal ones may not be non leukaemic lymphocytes. The occurrence of lymphocytes of normal and unchanged karyotype must always be remembered in the interpretation of the results of chromosome studies in the leukaemias.

*Acute Leukaemia*

The proportion of cases of acute leukaemia showing chromosome abnormalities has increased steadily as direct methods have replaced techniques involving preliminary culture. We are thus reminded of the uncertain basis for the belief that leukaemic cells show exuberant growth. Where normal marrow cells and peripheral blood lymphocytes are present they are usually able to overgrow leukaemic blast cells in culture. In studies using only direct methods for the examination of the bone marrow in acute leukaemia a very high frequency of chromosomal abnormality is found. FITZGERALD ADAMS and GUNZ (1964) studied 18 adult patients with acute leukaemia. They found abnormalities in all 11 cases studied by a direct marrow method compared with a very low yield of abnormalities in these and 7 other patients when peripheral blood cultures were used. In acute leukaemia of childhood, REISMAN, ZUELZER and THOMPSON (1964) using a direct marrow technique in the study of 13 patients found chromosome abnormalities in all. The only substantial series in which chromosome normality was found using a direct marrow method is that of SANDBERG, ISHIHARA, KIKUCHI and CROSSWHITE (1964). They studied 75 patients of all ages, treated and untreated, and found chromosome abnormality in 35 of them. There is no obvious explanation for the higher proportion of cases without abnormality in the series reported by SANDBERG and his colleagues. In their report chromosome normality clearly implies an absence of karyotypic change as well as a normal chromosome number. The small number of cells they were able to study in some cases has been pointed out (REISMAN *et al.*, 1964) but this is true only of a small proportion of their cases. In others their findings may be

due to remission after treatment, since not all of these patients were untreated, but this too can hardly explain all.

In the early years of chromosome studies in the leukaemias the difficulty of interpreting abnormalities in treated cases was discussed. The possibility of treatment inducing chromosomal abnormalities has probably been exaggerated, except perhaps in chronic granulocytic leukaemia treated by radiotherapy. In acute leukaemia ELVES, BUTTOO, ISRAELS and WILKINSON (1963) described gross chromosomal changes in peripheral blood cultures following treatment with 6-azauridine. Similar changes have not been described following treatment with 6-mercaptopurine. On the contrary REISMAN *et al.* (1964) studied 8 children with acute leukaemia before treatment and during remission induced by corticosteroid and 6-mercaptopurine. In all cases they found a marked change towards normal, with restoration of the normal chromosome number. In subsequent relapse the original abnormal modal chromosome number was generally restored although 2 of the cases showed additional aneuploidy. There have been no reports of studies before and during remission in adults with acute leukaemia comparable to those of REISMAN *et al.* in children. Their findings must be contrasted with those in chronic granulocytic leukaemia in children and adults in whom the frequency of the Philadelphia chromosome in the bone marrow is unchanged in remission (TOUGH, JACOBS, COURT BROWN, BAIKIE and WILLIAMSON 1963).

The results of chromosome studies in the leukaemias lend some support to the tripartite division of these diseases into acute, chronic granulocytic and chronic lymphocytic categories. The time has come for us to question the validity of the generic term, leukaemia, in every context in which we may use it. Only by doing so can we avoid the hazard of unwarranted generalization. Chromosome studies have provided no comparable support for the further division of acute leukaemia according to apparent cell type differentiation. Abnormalities have been found in cases described as granulocytic, lymphocytic, monocytic, myelomonocytic, stem-cell and erythroleukaemia. The only strong suggestion of correlation between the chromosomal abnormalities and possible cell-type differentiation has come from SANDBERG and his colleagues (1964). Their results suggest that in acute lymphocytic leukaemia the abnormal modal chromosome number is generally hyperdiploid, while in acute granulocytic leukaemia it is usually hypodiploid. The generalization

seems to be true for their lymphocytic cases with aneuploidy but 5 of their 14 cases of acute granulocytic leukaemia with aneuploidy also show a hyperdiploid modal number All of their lymphocytic cases with abnormalities were under the age of 15 and of their 14 cases of acute granulocytic leukaemia with abnormality only 2 were under that age. Thus as in many other situations, the separation of cases of acute leukaemia into adult and childhood types seems to be at least as helpful as classification according to cell type criteria, and is probably more realistic. Classification of acute leukaemia according to the age of patients has the added advantage that the allocation of every patient is beyond dispute.

It is obviously a matter of importance to establish whether or not these differences as regards ploidy between acute leukaemia showing lymphocytic and granulocytic differentiation may be found in patients of all ages. Apart from this possibility there is little evidence of either consistency or pattern about the results in acute leukaemia. No particular sort of chromosome abnormality is to be associated with erythroleukaemia or with either variety of so-called monocytic leukaemia. In 4 out of 41 cases of acute lymphocytic leukaemia SANDBERG ISHIHARA, KIKUCHI and CROSSWHITE (1964) found morphological abnormality of a chromosome of pair 1 KINLOUGH and ROBSON (1961) found an extra abnormal chromosome approximating to one of the group 13–15 in two cases of acute leukaemia in adults. The same workers have since found a third case with a similar but rather larger extra chromosome (ROBSON personal comm.) A comparable abnormality amongst others, has been described by KIOSSOGLOU ROSENBAUM, MITUS and DAMESHEK (1964) in a mongol with acute leukaemia. An extra chromosome of the 6–12 group was found by NOWELL and HUNGERFORD (1962) in 3 of the 9 adults with acute granulocytic leukaemia. WEINSTEIN and WEINSTEIN (1963) have reported the same abnormality in a child with acute granulocytic leukaemia, and STAFFORD KEMP and TAYLOR (1965) in one case of myelomonocytic leukaemia. In addition, MERCER, KELLER and LONSDALE (1963) and WARKANY SCHUBERT and THOMSON (1963) have each reported a mongol child with acute leukaemia having an extra chromosome of the 6–12 group in leukaemic cells, as well as constitutional trisomy 21 It must be pointed out that cases of acute leukaemia in mongols have been described as lymphocytic, granulocytic, monocytic and blast cell in type.

Apart from the mongols, these cases having similar chromosomal abnormalities have no other certain features in common. At present their common chromosomal abnormality may be regarded as having no special significance. If the manifold abnormalities found in the acute leukaemias arise at random then similarities must occur It is to be expected that similarities will involve the numerically largest group of chromosomes (6–12 + X) most often. BAIKIE, JACOBS, MCBRIDE and TOUGH (1961) suggested that chromosomal abnormalities might be more common in cases of acute leukaemia with a high probability of being radiation induced, in comparison with cases lacking a history of significant radiation exposure This suggestion arose from consideration of results obtained by earlier culture techniques and is now obviously fallacious. It remains possible that in their cases with a history of radiation exposure the conditions of culture were especially suitable for the leukaemic cells or in some way relatively inimical to normal cells in the mixed-cell populations they sampled and cultured.

Recently several groups of authors have reported unusual degrees of endoreduplication in cases of acute leukaemia (NASJLETI, WALDEN and SPENCER, 1965) This phenomenon is not specific for acute leukaemia and its significance is at present unknown.

Chromosome studies in individuals without leukaemia but with a known special risk of developing the disease have yielded most important results without revealing special pre-leukaemic changes, apart from the constitutional abnormality in mongols. When mongols develop acute leukaemia, trisomy 21 persists in the leukaemic cells. In addition, a proportion of cases have shown other chromosomal abnormalities. The proportion is, not unexpectedly related to the method of study employed, being high when direct marrow methods have been used and low using culture techniques. Most mongols with leukaemia showing additional chromosomal abnormalities have cells with more than 47 chromosomes. As has already been mentioned, in at least two cases these have included cells with an extra chromosome in the 6–12 + X group

Apart from mongols the other individuals known to have an increased liability to acute leukaemia are those who have been exposed to considerable doses of ionizing radiation, patients with polycythaemia vera and possibly people chronically exposed to benzene. It is still uncertain whether polycythaemia vera carries a greatly increased liability to acute leukaemia in the absence of radiation

exposure (MODAN and LILIENFELD 1964). After radiotherapy patients with polycythaemia vera (MACDIARMID 1965) and patients without diseases of the bone marrow (BUCKTON, JACOBS, COURT BROWN and DOLL, 1962) show both stable and unstable chromosome abnormalities in cultured peripheral blood lymphocytes. Recently similar chromosomal abnormalities have been described in persons previously exposed to benzene (TOUGH and COURT BROWN 1965). Although the mode of leukaemogenesis in these groups of people remains obscure it is highly suggestive of a causal relationship that all have some chromosomal abnormalities before leukaemia appears in a few.

*Chronic Lymphocytic Leukaemia*

Regardless of the methods used in attempts to study chromosomes in this disease no certain abnormality has been found. Marrow culture (BAIKIE, COURT BROWN, JACOBS and MILNE, 1959), peripheral blood cultures with added phytohaemagglutinin (NOWELL and HUNGERFORD 1964) and even direct marrow preparations (SANDBERG, ISHIHARA, MIWA and HAUSCHKA, 1961) have all yielded essentially normal karyotypes. In chronic lymphocytic leukaemia the peripheral blood lymphocytes do not divide without stimulation and divide only poorly with stimulation. All the tissues studied have been essentially mixed-cell populations and the metaphases examined may well have been from normal cells. Recent successes in the study of chromosomal changes in the lymph nodes in malignant lymphomas using short term culture without mitotic stimulation (SPIERS and BAIKIE, unpublished observations) suggest a need for similar studies in chronic lymphocytic leukaemia.

The status of the Christchurch chromosome (Ch¹) in chronic lymphocytic leukaemia remains uncertain. This was an apparent deletion of the short arms of a small acrocentric chromosome occurring as a constitutional abnormality in one family with two cases of chronic lymphocytic leukaemia in a sibship (GUNZ, FITZGERALD and ADAMS, 1962). In at least five other families with two cases of chronic lymphocytic leukaemia the same abnormality has not been found. Nevertheless, FITZGERALD (personal comm.) has recently found some new evidence which tends to associate the small acrocentric chromosomes with chronic lymphocytic leukaemia. In leukaemic males he found these chromosomes as a group to be

shorter than in normal males. The difference between leukaemic and normal females was not statistically significant. The possibility thus remains that one of the chromosome pairs 21 or 22 is associated with a special liability to lymphoproliferative disease. The original Christchurch chromosome may have been a fortunately extreme example which served to draw attention to the association.

### *Waldenström's Macroglobulinaemia*

Chromosome abnormality was first reported in macroglobulinaemia by BOTTURA, FERRARI and VEIGA (1961). Using a direct marrow technique they found a mixture of normal cells with 46 chromosomes and cells with 47 chromosomes having a very large abnormal chromosome. Since then at least seven other cases have been described with similar but not identical findings. In every case an extra chromosomal element has been present, apparently derived from one of the largest chromosomes. HAYHOE and HAMMOUDA (1965) have recently described negative findings in two more cases. They interpret these results as indicating that the chromosomal abnormalities are secondary to the disease. As with chronic lymphocytic leukaemia, negative results in Waldenström's macroglobulinaemia are not conclusive, in that the neoplastic lymphocytes may not have been represented amongst the cells in metaphase. In three more unreported cases chromosome abnormality involving a very large chromosome was found in two.

Results of chromosome studies in macroglobulinaemia seem to me to be second only to the Philadelphia chromosome in potential importance. The possibility of a more or less direct relationship between the chromosomal abnormalities of the very large chromosomes and the protein abnormality in the disease is high. This possible association is strengthened by the finding of a similar chromosomal abnormality in one case of cold agglutinin disease (LEVIN, HOUSTON and RITZMANN 1964). Every case seems worthy of as complete a cytogenetic study as possible, not only of the blood and bone marrow, but of lymphoid tissue as well.

### *Myelomatosis*

The position in myelomatosis has recently been reviewed by HAYHOE and HAMMOUDA (1965). They mention results obtained by various methods in a total of 17 cases. In 10 cases no chromosome

abnormality was found. In the other cases a variety of structural and numerical abnormalities were reported. A brief report by LEVIN *et al.* (1964) mentions studies on peripheral blood in 9 cases of myeloma, 8 with $\gamma$-type protein and 1 with $\beta$-2A protein. In 5 of these cases, including the patient with $\beta$-2A protein they found an abnormal metacentric or submetacentric chromosome in the same size range. A detailed report of these findings will be of obvious interest. The abnormality mentioned is very different to the gross aneuploidy and morphological changes in the reports reviewed by HAYHOE and HAMMOUDA.

Although early reports have been much less promising in myeloma than in macroglobulinaemia the same reasons make chromosome studies in the two conditions potentially rewarding The possibility of associating a chromosomal change with a metabolic abnormality in the neoplastic cell is obviously high Unfortunately all the familiar technical difficulties arise in myeloma. Sampling errors, lack of mitoses in direct preparations and selection against the myeloma cells in culture militate against easy and satisfactory results. In addition the mitoses that are obtained are often of notably poor quality

*Chronic Granulocytic Leukaemia*

The Philadelphia chromosome ($Ph^1$) has been found only in chronic granulocytic leukaemia. It has not been found in normal subjects as a constitutional chromosomal abnormality in individuals with or without chronic granulocytic leukaemia nor amongst the various chromosomal changes directly attributable to ionizing radiations. Furthermore, in chronic granulocytic leukaemia this specific chromosomal abnormality occurs only in the bone marrow and in blood cells derived from bone marrow It does not occur in the lymphocytes or the subcutaneous fibroblasts in patients who nevertheless have it in leukaemic cells (BAIKIE, COURT BROWN BUCKTON HARNDEN, JACOBS and TOUGH 1960) An abnormal chromosome indistinguishable from the Philadelphia chromosome has been found in only one individual without chronic granulocytic leukaemia. That was in a child with some of the stigmata of mongolism (DENT EDWARDS and DELHANTY 1963) The child had two normal chromosomes in addition and so was partially trisomic the leukaemic cells in chronic granulocytic leukaemia are partially monosomic for chromosome 21

Not all cases of chronic granulocytic leukaemia have the Philadelphia chromosome but exceptions are probably rare. It is now appreciated that the presence or absence of the abnormality can only be determined with confidence by the examination of bone marrow using a direct method without culture. Early reports of the absence of the abnormality in the peripheral blood in remission or acute transformation must be rejected in the light of the results of direct bone marrow examination (Tough, Jacobs, Court Brown, Baikie and Williamson 1963). In cultured material, certainly in the case of peripheral blood and probably in bone marrow too, the Ph¹ negative lymphocytes may replace the Ph¹-positive cells by the end of culture. It may readily be overlooked that in chronic granulocytic leukaemia in remission the peripheral blood may contain granulocytes which carry the Ph¹ but if these cells are mature polymorphs they will not be represented as dividing cells in a chromosome preparation. Apart from these pitfalls of method, cases of chronic granulocytic leukaemia apparently without the Ph¹ do occur.

Since the size of the deletion which accounts for the appearance of the Ph¹ chromosome may vary from case to case the possibility cannot be excluded that apparently Ph¹ negative cases in fact have very small deletions. Nevertheless, the best recognized group of patients without the abnormality are children with the so-called infantile type of chronic granulocytic leukaemia (Reisman and Trujillo 1963). These children may also be distinguished on clinical grounds by the frequency of lymphadenopathy, infection and haemorrhagic phenomena, and haemotologically by the low white cell counts, more frequent thrombocytopenia and monocytosis. Although Ph¹ positive cases of chronic granulocytic leukaemia in children tend to occur in older children the age distributions of the Ph¹-positive and the Ph¹-negative types overlap.

In adults, Ph¹ positive and Ph¹ negative cases are much less readily distinguishable on other than cytogenetic findings. Some Ph¹ negative cases may be quite typical of the disease without any unusual clinical or haematological features. Carbone, Tjio and Whang (1964) have compared 19 Ph¹ negative cases with 47 Ph¹-positive patients. They found that they differed significantly only as regards the lower median white cell and platelet counts and the shorter survival of the Ph¹-negative cases. Acute transformation appears to occur earlier than in the Ph¹-positive patients. The series studied by Carbone *et al.* consisted of all the untreated cases in 75

consecutive cases referred to them with a diagnosis of chronic granulocytic leukaemia. The proportion of Ph¹ negative cases is surprisingly high. In contrast, TOUGH (1965) has recently discussed 63 cases studied by the Edinburgh group since November 1961. There were only 3 Ph¹ negative cases, 2 in 55 without a history of radiation exposure and 1 in 8 with such a history.

It is of obvious importance that the Philadelphia chomosome does occur in cases of chronic granulocytic leukaemia which may be attributable to radiation exposure. It has been described in one closely exposed survivor of the atomic bomb explosion at Hiroshima who subsequently developed chronic granulocytic leukaemia (TANAKA, ITO, KAMADA and OKADA, 1963). Of course, in no single case can radiation induction of leukaemia be assumed however high the radiation dose. Consequently it is of great importance that in 7 of the 8 cases studied by the Edinburgh group the Philadelphia chromosome was present. All 8 had received X-ray therapy for ankylosing spondylitis. From TOUGH's comparison of the cases with and without a radiation history one probable difference emerged. She considered only patients in the chronic stage of the disease and compared the two groups as regards the chromosome findings in direct marrow preparations at first presentation. Five of the 7 with a radiation history had findings other than the simple Ph¹-positive state, including the one Ph¹ negative case. By comparison similar atypical findings were noted in 12 out of 50 cases without a radiation history. This difference is reminiscent of an analogous difference noted earlier (BAIKIE, JACOBS, MCBRIDE and TOUGH, 1961) between patients with acute leukaemia with and without previous radiation exposure. The analogy is incomplete and the earlier series was studied by less satisfactory methods but the similarity of findings should not be lightly dismissed.

One of the most remarkable features of chronic granulocytic leukaemia is the frequent occurrence of acute transformation of the disease (BERNARD, SELIGMANN and KVICALA, 1959). Any hypothesis concerning the cytogenetic findings in the disease must take account of this feature and the chromosomal changes associated with it. As an interim interpretation of the relationship of the Ph¹ chromosome to the natural history of chronic granulocytic leukaemia, the following may be suggested. The Ph¹ chromosome may arise from the action of radiation, a virus or viruses, a special liability of chromosome 21 to breaks in its long arms, or combinations of these factors.

It must either arise in a single cell which thereby acquires a growth or survival advantage, or if the Ph¹ positive cell has no such advantage the agent producing the first change must be capable of continued activity. By the time chronic granulocytic leukaemia is clinically or haematologically detectable all the marrow stem cells are Ph¹ positive. The loss of the genetic material from chromosome 21 has its main or sole effect on the granulocytic series. The stem cells of red cells and platelets also have the loss but only rarely show much effect. We do not know what determines the occasional presentation of chronic granulocytic leukaemia with erythraemia or thrombocythaemia (Tough, Jacobs, Court Brown, Baikie and Williamson 1963). Nor do we have any information about the duration of the period between the first appearance of the Ph¹ and clinical presentation. In any case, granulocytic differentiation is not obviously disturbed. The effects seem to be either on the mass of granulocytes produced, or on their life-span, the control of their release from the bone marrow, and some change allowing splenic haemopoiesis to occur. These familiar changes in the chronic stage of the disease may be reversed in a remarkable degree by treatment. Successful treatment has no obvious effect on the frequency of the Ph¹ chromosome in the bone marrow. No normal Ph¹ negative stem cells reappear except in the unusual situation where over treatment has led to a stage of marrow hypoplasia (Tough *et al.* 1963). The effects of treatment are on some consequence or concomitant of the appearance of the Ph¹ chromosome, and relapse occurs when treatment is stopped, if not before.

It now seems certain that the possession of the Ph¹ chromosome either makes a cell liable to additional chromosomal aberration or the mechanisms of its production can go on to cause other changes in cells already Ph¹-positive. Presumably these additional changes may occur at random although non-disjunction involving the Ph¹ itself seems especially likely to occur. The usual consequence of secondary changes is lethal for the cells in which they arise. Some changes must have a profound effect on granulocyte differentiation, on growth advantage, or on the normal ecological relationships of the stem cells of the three marrow elements without proving lethal. It is following such a change that acute transformation may arise. Just as we have learned to regard it as acute transformation instead of blastic change we might do well to widen the concept still further and call it metamorphosis, without implying the necessary occur

rence of any of the features we usually associate with acute leukaemia. Metamorphosis may be associated with the failure of previously effective treatment, as well as with anaemia, thrombocytopenia, thrombocythaemia, failure of orderly granulocytic differentiation, basophilia, lymphadenopathy and increasing splenomegaly.

It is obvious that not all the secondary chromosomal changes occurring in Ph$^1$ positive cells result in the metamorphosis of chronic granulocytic leukaemia. There have now been numerous reports of additional changes in patients still in the chronic phase of the disease. These Ph$^1$-positive cells with additional changes must have some advantage over Ph$^1$ positive cells without additional change. What is now certain is that these additional changes may have no adverse effect on the progress of the disease. Presumably the additional genetic change is usually irrelevant to the production, differentiation and function of marrow cells. The largest series of such cases has been described only recently (TOUGH, 1965). Amongst them were 3 males with Ph$^1$ positive cells lacking a Y chromosome. Similar cases have been described by others (ATKIN and TAYLOR, 1962; SPEED and LAWLER, 1964) and may be associated with long survival and especially favourable response to treatment. In these cases, further study may suggest that a factor on the Y chromosome is relevant to the natural history of chronic granulocytic leukaemia. Or can it be that Ph$^1$ positive cells of sex chromosome constitution XO are less liable to further chromosomal change than Ph$^1$-positive XY cells so that metamorphosis is less likely to occur? This and other problems may be answered by serial cytogenetic studies in chronic granulocytic leukaemia in relation to the natural history of the disease. In such studies, patients with a radiation history and others with additional chromosomal changes in the chronic phase will be of particular importance. The metamorphosis of chronic granulocytic leukaemia bears an obvious analogy to malignant change in neoplastic disease in general.

*Conclusion*

The chromosomal abnormalities already found in the leukaemias can hardly be irrelevant to their natural history whatever basic leukaemogenic agents may be discovered. The Philadelphia chromosome probably determines the character of chronic granulocytic leukaemia in the chronic phase and the additional chromo-

somal abnormalities its later natural history. More sensitive methods of studying chromosomes may enable us to detect aberrations analogous to the Philadelphia chromosome in the acute leukaemias, chronic lymphocytic leukaemia, myelomatosis and perhaps in many other neoplastic diseases. By the same means we may learn more about the metamorphosis of chronic granulocytic leukaemia and how it may arise.

The association of specific chromosomal abnormalities with metabolic changes may be possible in chronic granulocytic leukaemia and in Waldenström's macroglobulinaemia. Such studies have more than just haematological significance, as steps in the compilation of a chromosome map for man. The most thorough comparison of cells trisomic for chromosome 21 in mongolism with Ph[1] positive cells partially monosomic for the same chromosome is an obvious need. A real hope for the treatment of the leukaemias lies in the exploitation of specific changes while the cell population remains homogeneous.

The future does not only lie in more and more refined laboratory studies. There are many unanswered questions for the clinical haematologist, questions only likely to be answered by observation of particular patients over long periods. If all had an equal chance of developing leukaemia such studies would be particularly tedious and unrewarding. The opportunities for the critical observation or the particularly fruitful thought may be found in the more careful study of the individuals at especially high risk. Mongols, the irradiated, those exposed to benzene and patients with polycythaemia vera are already known. Who would be rash enough to say that others with a very special risk of developing leukaemia do not exist?


*Summary*

The short but fruitful history of chromosome studies in human leukaemia is briefly and critically reviewed. Attention is drawn to the limitations of current methods. The possibilities of associating morphological abnormalities of chromosomes with functional anomalies are emphasized. The results of cytogenetic studies in chronic granulocytic leukaemia are used to formulate an explanation for the natural history of the disease.

*Zusammenfassung*

Es wird eine knappe und kritische Übersicht gegeben über die kurze aber fruchtbare Geschichte der Chromosomenuntersuchungen bei menschlichen Leukämien. Es wird auf die Grenzen der gebräuchlichen Methoden hingewiesen. Die Möglichkeiten einer Zuordnung morphologischer Anomalien der Chromosomen zu funktionellen Stö-

rungen werden hervorgehoben. Die Ergebnisse zytogenetischer Untersuchungen bei chronischer myeloischer Leukämie werden herangezogen, um eine Erklärung für deren natürlichen Ablauf zu finden.


## *Résumé*

L'histoire courte mais abondante des recherches chromosomiales dans la leucémie humaine est brièvement résumée et commentée. L' ttention est attirée sur les limites des méthodes courantes. Il est insisté sur les possibilités d'associer les anomalies mor phologiques des chromosomes aux anomalies fonctionnelles. Les résultats des études cytogénétiques dans la leucémie myéloïde chronique servent à formuler une explication de l'histoire naturelle de cette maladie


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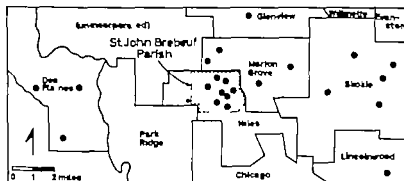



*Fig 1* Leukemia among children in Niles, Illinois, and vicinity 1956–1960. The solid dots indicate the cases of leukemia in children. (From ref. nr 6.)

is no higher frequency of leukemia among breast fed children than among "controls" which gives no support to a milk factor-like agent.

In contrast to these negative findings, reports about clusters or localized outbreak of leukemia among children have received great interest. Seemingly impressive in this regard were 8 cases of leukemia in a small community Niles in Illinois, outside Chicago (HEATH *et al* 1963) (Fig 1) The following observations were interpreted to mean that exposure to leukemogenic viruses led to leukemia in some children and antibodies in others without leukemic development. Blood was obtained from members of the leukemic patients families, and the sera were tested for antibodies to leukemic antigens including human leukemia and mouse leukemia. Antibodies against these antigens were present in one to three members of every family Positive reactors most often were the mother or a sibling closest in age to the patient with leukemia (Fig 2) Antibodies were not found in control sera or in the patients with leukemia (SCHWARTZ *et al* 1963) Moreover three of the children affected and the sibs of four others attended the same school and thus were considered to have had close contacts.

However the interpretation of the higher leukemia rate in Niles, as compared with those of the surrounding communities, poses several questionable points. It has not been shown that a cluster occurs more than would be statistically expected. Also, a cluster is not necessarily an evidence of a leukemogenic virus the etiological factors may be of other environmental types.

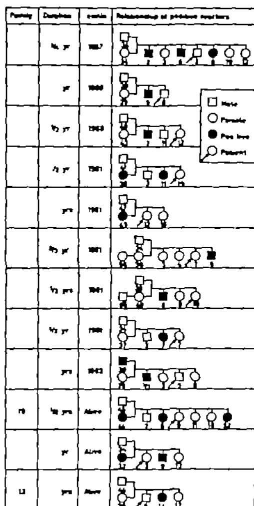


*Fig. 2.* Positive reactors against leukemic human antigens in serologic studies of families. Note the frequency with which the siblings closest in age to the patient have demonstrable antibodies. (From ref. nr. 7.)

Several other investigations of the same types (PINKEL *et al.* 1963, KNOX, 1964) and also studies of "spread" of leukemia in families, occurrence of leukemia in monozygous twins, etc. (MAC MAHON and LEVY 1964) have given similar and on the whole questionable results. There is as yet no substantial epidemiologic evidence that a viral agent plays a primary role in the origin of human leukemia.

*Immunology*

In the investigation of the leukemia "cluster" in Niles serological tests for antibodies against human and mouse leukemic tissue were put forward as evidence for the presence of the leukemia virus. G[illegible] *et al.* (1963) described immunologically specific antigens in leukemic tissue although without direct proof of the antigens being of viral origin. Fink *et al.* (1964) have used plasma from leukemic individuals and also the murine leukemia virus (Rauscher) to produce antisera in rabbits. After adsorption with normal human and murine tissues and labelling with fluorescein iso-

Comparison of findings in peripheral leukocytes of leukemic humans tested by two techniques—electron microscopy and immunofluorescence—using an antibody prepared against the concentrated plasma fraction of human leukemic patients

| Case No. | Diagnosis | Electron microscopy of pellet* | Fluorescence of peripheral blood leukocytes** |
|---|---|---|---|
| LIII | Stem cell leukemia | + | + |
| LVI | Acute myelogenous leukemia | + | + |
| HTL | Chronic myelogenous leukemia | + | + |
| P7 | Acute lymphocytic leukemia | + | + |
| P13 | Acute lymphocytic leukemia | + | + |
| P14 | Acute myelogenous leukemia | + | + |
| P23 | Acute lymphocytic leukemia | | + |
| P35 | Acute lymphocytic leukemia | + | + |
| P1 | Acute lymphocytic leukemia | | + |
| P4 | Acute lymphocytic leukemia | | + |
| P15 | Acute myelogenous leukemia | | + |
| P1[illegible] | Acute myelogenous leukemia | | + |
| P[illegible] | Acute lymphocytic leukemia | | + |
| P[illegible] | Acute lymphocytic leukemia | | — |
| P3 | Acute lymphocytic leukemia | | |
| P[illegible] | Acute lymphocytic leukemia | | + |
| P5 | Acute lymphocytic leukemia | | — |
| P7 | Acute lymphocytic leukemia | | — |
| P6 | Acute lymphocytic leukemia | — | — |
| P9 | Acute lymphocytic leukemia | — | — |
| P10 | Acute lymphocytic leukemia | — | — |
| P11 | Acute lymphocytic leukemia | | — |
| P[illegible] | Acute lymphocytic leukemia | — | — |

* These results were obtained from thin sections by electron microscopy through the courtesy of the investigator, Dr. [illegible] DALTON, National Cancer Institute.
** Buffy coat preparations from [illegible] normal individuals have been negative.

Fig. 3. Virus-like particles in plasma pellets and immunofluorescence reactions using an antibody prepared against the concentrated plasma fraction of human leukemic patients in leukemic patients. From ref. [illegible]

and SOUTHAM recently showed that *Herpes* virus exert a typical co-carcinogenic action in the production of squamous carcinoma in mice by polycyclic hydrocarbons.

NEGRONI also reported a marked cytopathogenic effect of the agent on human embryo cells in culture. This finding is difficult to interpret, however because the cytophysiological background of these morphological changes is not known. The *in vitro* test of the tumorigenic action of various isolated agents on human tissue must necessarily include such methods as, for instance those devised by WOLFF and further developed by BARSKI and WOLFF (1965) which directly demonstrate the infiltrative growth pattern of the transformed cells, with or without conspicuous cytological changes.

The numerous electron microscopical studies of sera, cells and *tissues from human leukemia will only be mentioned (Fig 5)*. Several workers report electron-dense particles bearing some morphologic

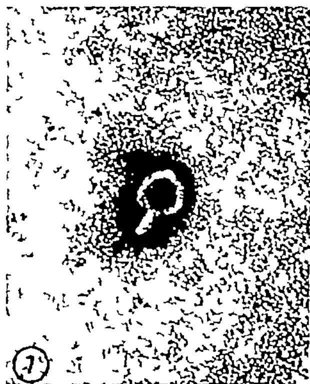


*Fig. 5.* Electron microscopy of peripheral blood pellet from 4-month-old female with acute stem cell leukemia. Magnification 49,500 times. (From ref. nr 26.)

similarities to viruses described in the avian and murine leukemias, but as stated from the group at National Cancer Institute the viral nature of these particles remains unproved and their relationship to the etiology of acute human leukemia is not established It is evident from many instances that electron microscopic appearance is an unsure criterion for a decision whether or not a particle is viral in nature.

Even if some forms of human leukemia will be proved in future to have a viral etiology recent research in epidemiology immunology and virology has so far not yielded any certain evidence that this is the case. The viral theory of human leukemia must still be handled with an open but critical mind by clinicians as well as by cancer research workers.


## *Summary*

The paper describes some epidemiological, immunological and virological evidences, which have been claimed to support the viral theory of human leukemia. For the present, however no definite conclusion can be reached, but further lines of research are indicated.

## *Zusammenfassung*

Es werden die epidemiologischen, immunologischen und virologischen Gesichtspunkte besprochen, die zur Stützung der Virustheorie menschlicher Leukämie herangezogen wurden. Im gegenwärtigen Zeitpunkt kann jedoch noch keine definitive Schlußfolgerung gezogen werden, vielmehr sind weitere Untersuchungen erforderlich.

## *Résumé*

Quelques faits épidémiologiques, immunologiques et virologiques sont décrits, faits qui ont servi à étayer la théorie virale de la leucémie humaine. Pour le moment, pourtant, aucune conclusion définitive ne peut être tirée Quelques indications sur les lignes à suivre dans les recherches futures sont données


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# On the Pathogenesis of Human Leukaemia

AA. VIDEBÆK

There is no convincing reason to believe that the development of leukaemia in man should be due to factors different from those which condition the development of leukaemia in fowls and a number of animal species. True, there is no direct evidence that human leukaemia is caused by virus. But as far as mice (26) and fowls (17) are concerned, the rôle of a virus is beyond doubt, and it is likely that in cattle leukaemia is also caused by a virus (31). The same applies to leukaemia in dogs (41) and cats (33) and in a preliminary paper reporting findings which have not yet been confirmed by others, it has been claimed that the so-called Burkitt lymphoma, occurring in African children, has been transmitted to monkeys (20). These lymphomas occur in a geographically delimited zone across Africa which has a minimum temperature of 15–16 C and a fairly high humidity. It would, all things considered, seem logical that human leukaemia too was virus-conditioned.

If parallels are to be drawn from experimental leukaemia in animals to human leukaemia, it must be assumed that although a virus may be a presupposition for its development, it is evidently not sufficient for leukaemia to arise or—put in another way—there may exist several, probably highly different, activators of a hypothetic latent leukaemogenic virus.

Of course, it is of interest to wonder why some individuals develop leukaemia, but if leukaemia is due to a widespread virus easily transmitted e.g. from mother to foetus or in some other way it is however even more peculiar that leukaemia does not affect a much larger number of individuals. Interest appears to have been centred almost solely upon the way in which leukaemia arises, while practically no heed has been given to the reasons why it does not manifest itself.

Undoubtedly inherited factors play a certain rôle in the development of leukaemia. By way of example, the incidence of spontaneous leukaemia in the inbred Street line of mice is very low only 1–2% of the mice dying of leukaemia. But in the also inbred AKA strain 50–70% of the mice which attain the age of more than 8 months will develop lymphogenous leukaemia. By exposing such mice to carcinogenic hydrocarbons (methylcholanthrene or 9 10 dimethyl-1 2-benzanthracene) the ability of an exogenous carcinogenic action to increase the number and hasten the development of leukaemia in mice has been demonstrated (40 55) ENGELBRETH HOLM (18) and others simultaneously (5 46) introduced the term acceleration to designate this phenomenon, as leukaemia was obviously not induced by this procedure, but a "spontaneous tendency to leukaemia was clearly accelerated

*Acceleration – Retardation*

Fig 1 is from LEFÈVRE s study on strain AKA (40) at the bottom the controls which developed 'spontaneous leukaemia at the

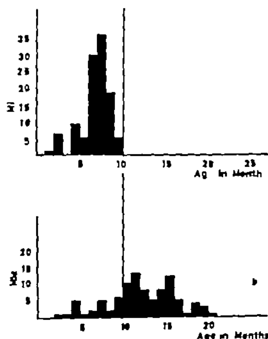



*Fig 1* Incidence and age distribution of *spontaneous* leukaemia in AKA mice, in controls (b) and mice painted with carcinogenic hydrocarbon ( ).

age plotted along the abscissa at a frequency shown on the ordinate. If the mice were painted on the skin with carcinogenic hydrocarbon, leukaemia developed before the age of 10 months in nearly 10 times as many as in the unpainted control series. The carcinogenic hydrocarbon obviously accelerated the leukaemia and increased its incidence.

Such an animal experimental model is well-suited for the investigation of factors which promote or inhibit the so-called spontaneous tendency to developing leukaemia. Among accelerators, apart from carcinogenic hydrocarbons, there are all types of electromagnetic and corpuscular radiation (23) and oestrogenic substances (24) at least in certain strains of mice.

Strangely enough, benzene, which is presumably leukaemogenic in man (2 48) has hardly been investigated for an ability to accelerate the development of spontaneous leukaemia in mice. Drugs such as sulphonamides, chloramphenicol, and phenylbutazone (6) have been in the limelight as possible leukaemogenic agents but the likelihood of such an effect has not been convincingly demonstrated. The same applies to cytostatics which are known to be at least teratogenic.

Among factors which antagonize the development of experimental leukaemia, cystein deficiency of the food and restriction of the caloric intake (56) may be mentioned. Testosterone does not only counteract the leukaemogenic effect of oestrogens, but inhibits the development of spontaneous leukaemia. Cortisone definitely inhibits lymphogenous leukaemia in mice (34) The general therapeutics are not included here. These findings from experimental leukaemia are mentioned in order to stress that possible a transmission of a leukaemogenic virus is an important or basic factor in the development of human leukaemia or certain types of it—just as with leukaemia in mice. However there must probably furthermore be leukaemia accelerating—activating or leukaemia increasing factors as well as leukaemia-retarding -antagonizing, and -reducing factors.

### *Statistics and Diagnosis*

Just a few words on leukaemia and statistics in general, not least because leukaemia statistics are often over-estimated. Statistics form the basis for observations of variations in incidence. Thus, statistics form the basis for detecting substances which may provoke

human leukaemia. But human leukaemia is a comprehensive and highly heterogeneous group of diseases which are also uncommon. Therefore the possibilities of statistics are often restricted by the small numerical size of a material and its inhomogeneity. Nevertheless, leukaemia is often treated as an entity which does give higher numerical values, but at the same time uncertain results, because the various types of human leukaemia must be assumed to represent different disorders having independent causes.

Chronic lymphogenous leukaemia is fairly well-defined, but does not appear to have a counterpart in experimental leukaemia. It is characterized by fully differentiated cells without chromosomal changes. The neoplastic nature of lymphogenous leukaemia as a tumor is fairly convincing in animals, but is it in man?

Chronic myelogenous leukaemia is also well-defined, characterized among other things by less differentiated cells and consistent chromosomal changes.

Acute leukaemia or stem-cell leukaemia seems to make up a group of its own which is in itself heterogeneous, but it is difficult to sub-groub as there are no reliable criteria, at least none on which there is general agreement. How often has not acute myeloblastic leukaemia been grouped with chronic myelogenous leukaemia and acute lymphoblastic leukaemia with chronic lymphogenous leukaemia—presumably with misleading results? It would be desirable if future statistics made a distinction at least between three main groups. Often, the statisticians appear to have been more keen on dividing their materials into the groups males and females which is far less importance, than on dividing them into these, evidently entirely different main groups of leukaemia.

A study on the epidemiology of leukaemia has come into vogue, and this appears to be a useful field. But it is evident that great caution must be exerted in assessing differences in incidences which often have been recorded under different conditions. Not infrequently careful statistical calculations are glaring contrast to the diagnostic uncertainty and heterogeneity of the groups. Statistics on leukaemia can never be more reliable than the diagnosis.

*Incidence*

It is beyond doubt that the leukaemias are being diagnosed far more often than before. This can probably not be demonstrated more strikingly than by figures from Great Britain published

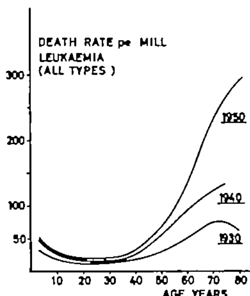



*Fig 2.* Schematic diagram of the very small increase of leukaemia in the lower age classes compared with the striking increase after the age of 50–60 years.

by DOLL (13) They show that the increase in the course of time has been equal for males and females. The mortality has doubled every 16 years.

There is much to indicate that by far the greater part of this increase is due to improved diagnosis. The increase affects all 3 main types of leukaemia—at least in the more advanced age groups In Denmark (26) and in Great Britain (9) the incidence of leukaemia in children increased up to about 1950 but not after that. In adults there is a very slow increase in the incidence with advancing age, but after the 50th–60th year the increase is abrupt (Fig 2) This late increase applies at leat in Great Britain to all three types of leukaemia, but in Denmark as well as in Great Britain primarily to chronic lymphogenous leukaemia. This late increase has been interpreted (6 13) as a sign of a more complete recognition of leukaemias in old persons, while the diagnostic facilities and rate of hospitalization as far as children are concerned reached almost a maximum during the forties (3 32)

From the point of view of research childhood leukaemia offers the advantage that this group does not at least include chronic myelogenous leukaemia or chronic lymphogenous leukaemia of any

numerical significance. The group is no doubt inhomogeneous, but far less so than leukaemia in adults. Therefore, a relatively great emphasis may be laid upon statistics on childhood leukaemia. Two practical conclusions appear to be justified from these statistics, viz. (1) that there is a so-called childhood peak (2) that the incidence of acute leukaemia in children no longer seems to be increasing

This childhood peak which was noticed in Great Britain in 1955 and which is incidentally most marked in boys, has been confirmed from a number of other countries, among others U S.A. (64) where it was limited to the white population and it is also distinctly apparent from the Danish vital statistics. The peak is at

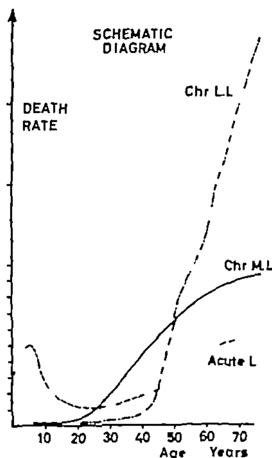



*Fig. 3.* Schematic diagram showing relative frequency of three main types of leukaemia. Unlike the chronic types acute leukaemia appears biphasic.

the age of 3 or 4 years as seen in Fig 3 The explanation of this "childhood peak and the increasing incidence of acute leukaemia through adults age, that is a biphasic age distribution of acute leukaemia, may be that two different diseases are included in the group of acute leukaemia or (taking a parallel in the acceleration experiments on mice) that the leukaemia accelerating factors—which need not be identical for the two peaks of the curve—are active during two periods.

In a Danish series of 514 cases of acute leukaemia in children it was (32) calculated that the risk for a child in Denmark to develop acute leukaemia during the first 14 years of life is 65/100 000 for boys and 46/100 000 for girls. The basis of this risk is entirely unknown, but the risk does not appear to be constantly increasing as was believed during a certain period.

Leukaemia is uncommon in East African children, but they develop a large number of solid lymphomatous tumours which nearly always remain non-leukaemic (11) and chloroma (12) seems remarkably common in Uganda. However leukaemia is common in African adults, especially females. The explanation of this rather remarkable difference in the incidence of leukaemia in children and adults is open to discussion. It is suggested that either African children possess som factors of resistance to childhood leukaemia or the provoking agents for adult and childhood leukaemia are entirely different.

### *Factors Influencing Incidence*

On the whole, we know very little about which factors are of significance to the development of human leukaemia. The best known ones are electromagnetic and corpuscular radiation. The clinical experience in this respect may be summed up as follows

1 Survivors of the atomic bomb explosions in Japan 1945 showed a maximum incidence of leukaemia in 1951 *i.e.* about 6–7 years after the exposure. Out of 92 cases of leukaemia 52 were of the acute type, 39 of the chronic myelogenous type and only one got chronic lymphogenous leukaemia (49) The explanation of this rare occurrence of chronic lymphogenous leukaemia in this material is perhaps connected with the remarkably rare occurrence of this type of leukaemia in the Far East.

2. In spite of all, it does not appear entirely convincing that the incidence of leukaemia is still increased among radiologists.

However according to data from U.S.A. (16) doctors develop leukaemia almost twice as often as the general population, and the incidence among radiologists appears to be about 8 times that among other members of the profession. Such an increased risk was not found in a study of the incidence of leukaemia among British radiologists (8) and incidentally many radiologists are to a great extent not exposed to X rays.

3 The results found in patients treated with X rays to the spine for ankylosing spondylitis (7) suggest that 1 rad produces about one case of leukaemia per 1 million individuals per year for at least 6–7 years. Out of 41 cases of leukaemia recorded in the study 30 patients developed acute leukaemia, and it is noteworthy that 7 of these were classified as acute monocytic, 14 as acute myelogenous leukaemia. Six patients developed chronic myelogenous leukaemia, and again only one patient chronic lymphogenous leukaemia. This distribution differs much from the distribution of leukaemia in the general population The over-all incidence rate in the whole group was calculated as being 10 times increased. The latent period varied from seldom, less than two years to 10 years and was, on an average, 6 years as in the survivors of the atomic bomb explosion.

4 X-ray irradiation of an enlarged thymus in children appears to induce not only thyroid cancer but it may increase the incidence of leukaemia to about 5 times that otherwise expected. The type of leukaemia is the acute one (37 61) The average latent period was again about 6 years (1–13 years)

5 For some time it has been discussed whether irradiation of the pregnant uterus could induce leukaemia in the infant. Most figures are small, and some investigations speak for (59 22) and others against (10 30 42) such an increased risk. However MACMAHON's painstaking study in 1962 (43) appears to show that in fact there is a moderate leukaemogenic effect of intrauterine exposure to diagnostic X rays, such as pelvimetry examination for twins, placentography gastrointestinal examination, and intravenous pyelography The finding of 47 cases of acute leukaemia in irradiated children was about 40% higher than in non irradiated children.

To these clinical finding it may be added that radiation is known to be accompanied by a shower of chromosomal changes, but there is no guarantee that radiation induced leukaemia is

caused by these changes. There are several substances which may entail chromosomal abnormalities, without there being any evidence of their also being leukaemogenic.

It may be concluded that *therapeutic* irradiation convincingly increases the risk of acute leukaemia and chronic myelogenous leukaemia. *Diagnostic* irradiation of the foetus moderately increases the risk for the child to develop acute leukaemia. Radiation-induced or accelerated leukaemia is predominantly of the acute or the chronic myelogenous type. This conclusion is supported by the observation that patients with chronic myelogenous leukaemia have a more significant history of exposure to radiotherapy and diagnostic X ray examinations than patients with chronic lymphogenous leukaemia and lymphosarcoma (21, 27, 58). It must be assumed, however, that diagnostic radiography is responsible for only a very small proportion of the total cases of leukaemia.

*Table I*

Birth order of 1747 cases of childhood leukaemia (MacMahon and Newill, 1962)

| | | | Normal distribution |
|---|---|---|---|
| 712 cases | 1st birth | 41 % | 37 % |
| 553 cases | 2nd birth | 32 % | 32 % |
| 267 cases | 3rd birth | 15 % | 17 % |
| 116 cases | 4th birth | 6 ½ % | 7 % |
| 99 cases | 5th and later birth | 5 ½ % | 7 % |

In connection with the discussion about the development of leukaemia in children, it is interesting that with excessive maternal age (with which mongolism is associated too) there also seems to be an increased risk of acute leukaemia in the child (44). The parity has been stated to be a factor (44), leukaemia apparently being a little more common in first born children (Table I).

The difference from the normal distribution should be modest but significant. The risk has been stated to increase with the mother's age, but to decrease with the number of previous, live births.

## *Chromosomal and Extrachromosomal Factors*

Since the viral aetiology of mouse leukaemia was discovered (26), the interpretation of the rôle of inherited factors in the development of mouse leukaemia has altered to some extent. However

there is hardly any doubt that genetic factors also play a rôle in the *development of mouse leukaemia*. In man, the factors relating to possible genetics, and infection are far more difficult to elucidate, partly because leukaemia is a rare disease—which for instance in Denmark is responsible for less than ½% of all deaths—and partly because human populations are genetically very inhomogenous. Lastly there might be in man a question of so-called "vertical" transmission of virus through the germ cells from one generation to the next, simulating heredity Moreover there is a possibility that the virus may induce chromosomal changes. Familial clusters of leukaemia may of course also be due to other laws of spreading infection.

Let me merely recapitulate a few findings of interest in the discussion on the rôle of chromosomal and extrachromosomal factors in human leukaemogenesis.

1 Family concentrations of leukaemia, with up to 5 leukaemic members in the same family Such a large number of cases of leukaemia may even occur within the same sibship (1 29 52) An occurrence in several successive generations has also been reported repeatedly

2. A proband study revealing 8% of 209 leukaemic patients with a family history of leukaemia, compared with ½% among 200 control families (62)

3 Significantly excessive incidence of cancer among the relatives of leukaemic patients (50 63) Among 4041 relatives of the 209 leukaemic patients there were 319 cases of cancer among the 3641 relatives of the 200 control probands only 218. The higher incidence in the patient material was demonstrable in all categories

*Table II*

Cancer incidence and risk for relatives of 209 leukaemia patients and 200 probands (VIDEBÆK, 1947)

| | Probands | Relatives | Cancer Cases | Cancer Incidence | Cancer Risk | |
|---|---|---|---|---|---|---|
| Chr lymph. leukaemia | 68 | 1429 | 131 | 9.2 | 30 | 31% |
| Chr myel. leukaemia | 56 | 1086 | 94 | 8.7 | 31 | |
| Acute leukaemia | 85 | 1503 | 94 | 6.3 | 33 | |
| total | 209 | 4018 | 319 | 7 7 | | |
| Controls | 200 | 3641 | 218 | 6.0 | 22% | |

of relatives. It was thus demonstrated that among the relatives of the leukaemic patients there was a significantly excessive incidence of cancer as a whole, due to a high incidence of all forms of cancer (Table II) Cancer risk means the probability of the relatives getting cancer if they live throug all age groups. This cancer risk was calculated for each category of relatives of the leukaemic probands, and the values were found to be between 23 and 48% with an average of 30% 31% and 33% for the three types of leukaemia. For all relatives of the 200 control probands the risk of getting cancer was significantly lower i.e. 22%

4 A twin study (44) with a probable concordance rate as high as about 25% for leukaemia in sets of monozygous twins. Out of 72 sets of twins with at least one member affected by childhood leukaemia, 5 sets showed both members affected by leukaemia—a finding highly suggestive of the influence of genetic factors.

5 The strangely rare occurrence of chronic lymphogenous leukaemia in the Far East (57)

6. a) An excess incidence of leukaemia in mongoloid children and excess incidence of mongolism among leukaemic children.

b) Major congenital defects, other than mongolism, were about twice as common among leukaemic children as would have been expected (47)

c) An increased incidence of mongolism among the siblings of leukaemic children (44)

d) An excess of cancer and leukaemia among the siblings of leukaemic children (47)

e) The frequency of mongolism as well as of childhood leukaemia increases with the *maternal age at birth of the child*, as

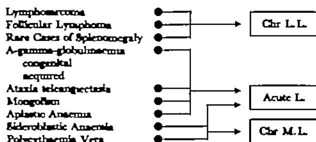



*Fig 4 Preleukaemic states* in relation to types of leukaemia.

already mentioned. However it is difficult to decide whether these finding are based upon pre- or postnatally factors.

f) In practically all patients with chronic myelogenous leukaemia there is a characteristic chromosomal change (the $Ph_1$ chromosomal defect)

It is still too early to assess the ever increasing number of observations reporting clusters of leukaemia. Some of the reports are based on very small materials, but a few larger series are available already indicating a focal distribution of acute leukaemia unlike chronic lymphogenous leukaemia (60) and that leukaemia has a tendency to occur in the general neighbourhood of previous cases (51) Certain materials indicate, in a rather convincing manner seasonal variations in the incidence of acute leukaemia with one or two annual peaks (36, 39) It seems reasonable to interpret such a geographic and seasonal accumulation of the cases in favour of a possible viral aetiology of human leukaemia.

*Preleukaemic States*

The type of these conditions appears to differ somewhat for the different types of leukaemia (Fig 4) but in the great majority of patients with leukaemia the pathogenesis is still entirely unknown. A few cases of chronic lymphogenous leukaemia have been preceded by either lymphosarcoma or follicular lymphoma which had been present for several years. Possibly genetics play a more important rôle in the development of lymphogenous leukaemia than of the other types of leukaemia. In this connection it is of particular interest that patients with congenital or acquired immunoparesis develop various malignant lymphomas with striking frequency and in one case leukaemia has been observed. These cases of immunoparesis are rare cases of congenital or acquired agammaglobulinaemia (33) This group in fact also includes cases of ataxia teleangiectasia, a familial disease consisting of progressive ataxia, oculo-cutaneous teleangiectasia beginning in early childhood and—of particular interest in this connection—immunoparesis, especially gamma-A deficiency and therefore repeated, severe infections. Although a large number of these children succumb to infections, there were nevertheless among approximately 125 known cases not less than 8 who died of lymphocytic or reticular malignancy (25) If human leukaemia and malignant lym

phomas are caused by virus, there is nothing surprising in the fact that these very patients with immunoparesis so often prove to develop leukaemia and lymphomas.

As already mentioned, mongoloid children develop acute leukaemia 10–20 times as often as healthy children, but it is a moot point whether mongolism can be considered a pre leukaemic condition. Some cases of aplastic anaemia (15) and very rare cases of chronic thrombocytopenia and neutrocytopenia may within months or years, develop acute leukaemia or chronic myelogenous leukaemia, and 15–20% of patients with sideroblastic anaemia (4) develop either chronic myelogenous leukaemia or erythroleukaemia (28) In polycythaemia vera treated with radioactive phosphorus there is a high incidence of acute leukaemia (38) but it is uncertain whether patients with untreated polycythaemia vera may occasionally develop acute leukaemia as a part of the natural history of polycythaemia. If so it appears to be very rare, and the development takes at least a very long time.

The transition of these conditions into leukaemia is rather baffling, but when considering leukaemia as a whole, these possibilities count but little. Nor does radiation induced leukaemia contribute materially to the total number of cases.

Thus, on the whole the aetiology and pathogenesis are entirely unknown. Intensive study not least of experimental leukaemia in animals, has greatly extended our knowledge, and it is to be hoped that soon it will lead to the final goal which is an effective treatment or perhaps even an effective prophylaxis of the human leukaemias.


### *Summary*

The pathogenesis of human leukaemia is reviewed in the light of leukaemia accelerating and retarding factors known from experimental leukaemia in mice. Some of the difficulties on leukaemia statistics are underlined; first of all the variations of incidence according to sex, age and geography are mentioned critically as well as the importance of electronic and corpuscular radiation and also of hereditary (or better familial) factors. Finally are the so-called preleukaemic states briefly discussed.

### *Zusammenfassung*

Es wird eine Übersicht gegeben über die Pathogenese der menschlichen Leukämie im Hinblick auf leukämiebeschleunigende und -verzögernde Faktoren, wie sie von der experimentellen Leukämie bei Mäusen bekannt sind. Einige Schwierigkeiten der Leukämiestatistiken werden hervorgehoben. Vor allem werden von Geschlecht, Alter und geographischen Einflüssen abhängige Variationen des Befalles kritisch gewürdigt,

sowie die Bedeutung elektronischer und korpuskulärer Strahlen und der hereditären (oder besser familiären) Faktoren. Schließlich werden die sogenannten präleukämischen Zustände kurz diskutiert.

## Résumé

La pathogénèse de la leucémie humaine est revue à la lumière de facteurs connus de la leucémie murine expérimentale, facteurs accélérant ou retardant la leucémie. Quelques unes des difficultés des statistiques sur la leucémie sont soulignées avant tout les variations de l'incidence selon le sexe l'âge et la géographie sont mentionnées de façon critique ainsi que l'importance de radiations électroniques et corpusculaires et de facteurs héréditaires (ou mieux familiaux). Enfin les états dits préleucémiques sont brièvement discutés.

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# Structure and Heterogeneity of Antibodies*

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The concept of the heterogeneity of antibodies – that the antibody formed even to a single antigen is not homogeneous but consists of populations of molecules with different physical, chemical or immunochemical properties – goes back in time to the discovery of cross reactions with antisera to highly purified protein or polysaccharide antigens (cf. LANDSTEINER 122) and the recognition that antibodies formed to a single antigen could consist of more than a single class of protein was a consequence of the first applications of the ultracentrifuge (91, 104) and of the Tiselius electrophoresis apparatus (220) to study potent antisera and purified antibodies produced in various species. These and other earlier studies also demonstrated changes in the electrophoretic and ultracentrifugal patterns of these components during the course of immunization of horses with pneumococci (104) and with various antitoxins (112, 227). The state of protein chemistry during this period, however, made it impossible either to appreciate the true complexity of the problem, or to interpret correctly the significance of the changes in physicochemical properties during continued immunization, or to provide any reasonable correlation with the various biological manifestations of antibody activity.

The extraordinary progress in this area, especially during the past decade, has provided new insights and has made it possible to attack the structure of the immunoglobulins and of antibodies in a direct and vigorous manner. One should recognize that immunochemical progress was substantially hastened by developments in the fields of protein structure, of enzyme-substrate inter-

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actions, of chromatographic and other physicochemical methods of purifying and characterizing proteins, peptides and other substances and on the genetic basis of protein synthesis. One of the most gratifying concepts to emerge has been the relationship of the myeloma globulins and Bence Jones proteins to the immunoglobulins, a parallelism so extensive that it lends great confidence to the formulation of structures for the immunoglobulins and provides information on their cellular origins and functions.

It seems most reasonable to attempt a comprehensive description of the various parameters of our present knowledge of the heterogeneity of the immunoglobulins and antibodies and indicate the major implications rather than attempt a historical survey of the subject. Many important reviews (18 31 45 60 89 90 92, 94 192, 206, 230) several symposia (77 135 215a) and books (75 76, 106 178) have been written recently on various aspects of this subject. This review will deal predominantly with human immunoglobulins and antibodies, but will mention relevant data obtained in other species.

Our present insight into the heterogeneity of the immunoglobulins dates from the introduction of immunoelectrophoresis by GRABAR and WILLIAMS (78 cf 75 76 92) for the study of the serum proteins. This technique permitted recognition in normal sera from a wide variety of species of three classes of proteins originally termed gamma, $\beta_2$M and $\beta_2$A globulins and now known as the immunoglobulins and designated $\gamma$G $\gamma$M and $\gamma$A or IgG IgM and IgA respectively (235) The $\gamma$G and $\gamma$M globulins correspond to the two proteins (S 7 and S 19) previously shown to be associated with antibody activity and the $\gamma$A protein (S 7–13) was a new class of immunoglobulin, not previously recognized in most species of antisera but having been studied earlier as the T component of horse antitoxic sera (112, 227) $\gamma$A globulin is the predominant immunoglobulin in parotid saliva, colostrum, lacrimal secretions (222, 223) and in nasal (6, 186) and bronchial fluids (111) and $\gamma$A antibodies have been found in those fluids (cf 222 223) Proteins of lower molecular weight antigenically related to the immunoglobulins appear in normal plasma and urine (16 58, 59 232 233) Another major advance by immunoelectrophoresis was the demonstration that the $\gamma$G globulin was an extremely heterogeneous population of molecules with different net charges in that the $\gamma$G line in immunoelectrophoresis extended from the slowest

gamma region up to the $\alpha_2$-globulin In some instances the gamma globulin line was double throughout its entire length (41 75 76, 92 115) Heterogeneity of γG globulin had also been established previously by studies on boundary spreading (1) and by the demonstration by JAGER *et al* (103) that α-globulin fractions carefully separated in the Tiselius electrophoresis apparatus reacted with antisera to γG globulin but immunoelectrophoretic analysis permitted this heterogeneity to be studied very easily and rapidly

In various animal and most recently in human sera a fourth class of immunoglobulin has been described. In the human sera the immunoglobulin is called IgD or γD (193 194) but to date no purified antibodies have been found to be of this type.

The host of distinct proteins isolated in large amounts from the sera of individual patients with multiple myeloma and macroglobulinemia have been found to fall into these four classes of immunoglobulins. These pathological globulins are generally much more homogeneous than the immunoglobulin itself and appear to represent the products of a single cell line, the immunoglobulin synthesizing abnormal cell. Each of these myeloma globulins differs both from the whole immunoglobulin of the class to which it belongs and also from proteins of other pathological sera belonging to the same class. Thus individual myeloma globulins of the γG variety show a very narrow range of mobility as compared with the extremely broad range of γG globulin.

Also associated with diseases such as multiple myeloma are the urinary Bence Jones proteins which also differ from one patient to another These have been found to fall into two broad types which are antigenically distinct, originally called types I (B) and II (A) (114) but now termed K and L (235) Using antisera to the K and L Bence Jones proteins, it has also been found that the serum myeloma globulins and pathological macroglobulins also fell into the same two types, each patient producing a myeloma globulin or Waldenström macroglobulin corresponding in type to his Bence Jones protein (49 136) About 60% of normal γG molecules are of type K and 30% of type L (46, 137) which corresponds closely to the relative incidence of myeloma cases of these types (49 136)

Tests with type K and L antisera have shown that the three major immunoglobulin classes may each be subdivided. Some of the molecules of each class possess K determinants and others pos-

sera L determinants (46 136) Thus six subclasses of immunoglobulin become recognizable. The IgD myeloma protein was of type L (193) adding a seventh class. The presence of type K and L molecules provided an explanation (47 137) for the finding that with some antisera the γG line was double (41 115) Recently a third antigenic specificity has been described for these proteins (24) and a triple line has occasionally been reported on immunoelectrophoresis (10)

When purified γG antibodies were studied they were found to fall into both classes (138) Antidextran and anti A sera from individuals varied in the proportion of K and L containing molecules present most of the antibodies were close to the 60 30 ratio found for the incidence K and L γG molecules in normal serum but one anti-A serum contained 90% of molecules with type K and only 10% with type L determinants one antidextran and another anti-A had 80% K and 20% L molecules (118) By contrast, however 15 cases of purified cold agglutinin studied in several laboratories (63 118) showed only K determinants and a variety of erythrocyte autoantibodies of the γG variety had exclusively or predominantly K determinants (127) Thus with respect to K and L molecules most antibodies are mixtures. The occurrence of anti thyroid antibodies in each of the six immunoglobulin classes γG K and L, γA K and L and γM K and L has been described (48) Blood group antibodies, notably anti A and B have been found to fall into the three immunoglobulin classes (143 189) anti Rh occurs in the γG and γM but there are differences as to its presence in γA (176 181) Other antibodies are also γA globulins (195) Blocking antibodies in human brucellosis sera have been found to be γA globulins (236)

A third parameter of heterogeneity of the immunoglobulins was observed by EDELMAN (37) on electrophoresis in starch gel after reduction of γG globulin with mercaptoethanol in 8 M urea and alkylation with iodoacetamide. If the electrophoresis was carried out in urea formate at pH 3 0 whole γG globulin gave two diffuse bands, the slower migrating one called the heavy (H) chain and the more rapidly migrating one the light (L) chain band. When myeloma globulins were examined under these conditions a few sharp L chain bands were observed, the number generally varying from one myeloma globulin to another again indicating that they represented selected populations of molecules synthesized by a

much smaller spectrum of cell types than whole γG globulin Bence Jones proteins similarly treated showed L chains identical to those in the myeloma globulin from the same patient (40) Purified antibodies obtained from guinea pigs (38, 39) and from humans (42) also showed similar sharp L chain bands indicating that they too came from selected cell populations. Among the guinea pig antibodies some suggestion of the association of specificity with the L chain bands was noted but this was not apparent among the human antibodies. With the latter differences in the L chain bands were found for five antibodies to different antigens produced in one individual (42) However the L chain bands also varied in number intensity and mobility when antibody to the same antigen (dextran) was obtained from several individuals the very simple nature of the antigenic determinant involved (α1 6 linked chains of glucose) also is consistent with responses of different cell populations to the antigen.

A substantial increase in the resolving power of starch gel electrophoresis was achieved at alkaline pH. COHEN and PORTER (30) found that under these conditions normal human γG globulin gave 10 L chain bands rather than the diffuse single band obtained at acid pH and individual myeloma globulins showed only relatively few bands. Of especial significance for heterogeneity of the immunoglobulins was the finding that individual myeloma globulins gave single bands corresponding to one or another of the 10 L chains but with either K or L determinants. One would infer therefore that, in normal γG globulin, each of the 10 L chain bands contained some molecules with type K and others with type L determinants. Thus there would appear to be at least 20 varieties of γG globulin based on these two parameters.

A variety of other observations over the years had an important bearing on the heterogeneity of the immunoglobulins, and especially of γG globulin, and also made possible the formulation of a structure for γG globulin. For over 30 years it has been known that digestion of horse antitoxic globulins with pepsin produced a molecule with intact antitoxic potency but with only ⅔ of the molecular weight of the native antitoxin (166 cf 106) and earlier studies with papain digested antitoxins (168 170) showed splitting into half and quarter molecules only one of the half molecules flocculated with toxin. In 1950 PORTER (172) obtained a fragment on papain digestion of rabbit antiovalbumin which inhibited pre-

cipitation of intact antibody by ovalbumin but it was not until 1958 when rabbit $\gamma G$ globulins and antibodies treated with papain were found to show three peaks by chromatography on CM cellulose that real insight into the structural significance of these effects was realized. The papain had been activated with cysteine and it was at first thought that in addition to the splitting of peptide bonds, reduction of a disulfide bond had occured to give three fragments (173 174) Two of these fragments, now termed Fab (235) when obtained from antibody $\gamma G$ each possess a single antibody combining site, while the third fragment Fc (235) was inactive. The Fab fragments were of two types e.g the first two peaks (I and II) obtained on CM cellulose chromatography of rabbit $\gamma G$ globulin and it was subsequently shown (164 208 215 229) that each molecule was built up of two identical Fab (I or II) and of one Fc fragment.

Investigation of the effects of pepsin on rabbit antibodies showed that the Fc fragment was destroyed to yield a bivalent antibody on further treatment with cysteine, a single disulfide bond is reduced and the molecule splits into two univalent fragments (151) termed Fab (if pepsin is used) Further studies by NISONOFF and coworkers (149 150) showed that on reoxidation of the SH to SS bonds, a bivalent antibody which precipitated could be formed. If univalent antibody fragments to two different antigens were mixed and reoxidized some bivalent antibody with one grouping directed toward one and the other toward the second antigen could be formed (149) The papain Fab fragments neutralized type II poliomyelitis and western equine encephalomyelitis viruses *in vitro* but not *in vivo* (33) Neutralizing ability of pepsin digested rabbit anti-phage antibody was less than that of intact $\gamma G$ antibody and reduction with cysteine to the Fab fragments caused further reduction in potency (74) The binding of pepsin digested 5S fragments of rabbit anti A for the A red cell was about the same as that of intact antibody Reduction to the 3.5S Fab fragment caused a marked drop in association constant. Recombination of the univalent 3.5S fragment with a normal 3.5S $\gamma G$ fragment did not increase the binding but specific recombination to produce a 5S bivalent antibody gave the same association constant as originally found (79) Further studies in this area are needed.

An important development was the finding (55) that reduction of $\gamma G$ globulin with 0 75 M mercaptoethanol in tris buffer at pH 8.2 and chromatography on G-75 or G-100 Sephadex in cold

N acetic or propionic acids yielded two peaks originally designated A and B which correspond to the heavy and light chains respectively The molecule is made up of two heavy and two light chains having molecular weights of about 55 000 and 20 000 held together by disulfide bonds. There is some uncertainty as to the number of interchain disulfide bonds holding the molecule together Three appears to be a minimum number since it has been shown with rabbit γG that ⅓ to ½ of the molecules are split into half mole cules by 0.015 M mercaptoethylamine at pH 5 if treated with iodoacetamide, a value of one S-carboxymethylcysteine e.g one SH group per 75 000 molecular weight is obtained (165) However the 4 chain structure of Porter originally indicated five disul fide bonds and were there only one S-S bond holding the heavy chains together the Fc fraction should be split into half molecules without further reduction of S-S bonds. This does not appear to be the case (139) These uncertainties in the S-S bonds may also be a reflection of heterogeneities in γG globulin (31) The various S-S bonds in the molecule apparently are not equally labile to re duction. The four chain structure is not without its limitations and several groups (31 61) have pointed out that a six chain structure in which some as yet unknown linkage connects the two portions of the H chain in the vicinity of the site of papain cleavage would be more consistent with some of the biological observations. Either the four or six chain structure serves as a very convenient framework for discussing further heterogeneities. Analyses of heavy and light chains of human γM and γG globulins for amino acids showed the latter to be similar for both classes of immunoglobulin while the former were quite different (27)

In addition to the large number of L chains mentioned above each with K and L determinants, the heavy chains also are heterogeneous (175) TERRY and FAHEY (219) using antisera to normal γG globulin prepared in rhesus monkeys (cf 35) and GREY and KUNKEL (81) using rabbit antisera to whole γG or myeloma γG globulins have shown that at least 4 kinds of heavy chains exist in γG globulin. Moreover a number of cases characterized by the presence of H chains in the urine and serum have also been described (62 159) and these have shown two distinct specificities. Two of the heavy chain subgroups were found in all three laboratories. How many kinds of heavy chains will ultimately be described for γG globulin remains to the future. The other classes of im-

munoglobulin are generally considered to differ from $\gamma$G globulin *in their heavy chains which generally appear to be unrelated immunochemically to those of* $\gamma$G globulin.

Immunoglobulins of the various species studied including rabbits, mice and humans possess certain genetic markers recognized by the use of specific antisera. These allotypic or isoantigenic markers were first discovered in rabbits by OUDIN (160) by immunizing rabbits with the serum of other individual rabbits two allotypic loci have been recognized and widely studied (36 50 128 160) In man, the Gm (84) and Inv (191) groups represent comparable systems of allotypes (66, 84 214) Certain selected normal human antisera or the sera of certain patients with rheumatoid arthritis detect isoantigenic differences among the serum $\gamma$G globulins of various individuals. The usual system employed is an Rh (D+) erythrocyte coated with human anti-D (66, 84 191 214) These coated erythrocytes will be agglutinated by the normal or rheumatoid serum. The detection of the appropriate Gm factor in various normal human sera is performed by titrating the capacity of the sera to inhibit agglutination in the system. If the anti-D used to coat the erythrocytes contains the Gm and the agglutinating rheumatoid or normal serum contains the appropriate anti-Gm specific antibody the system will be specific for Gm In human immunoglobulins the major allotypic determinants are Gm $Gm^b$ $Gm^{b2}$ or $Gm^f$ and $Inv^a$ and $Inv^b$ There are a variety of subgroups related to Gm (cf 214) At least two loci are involved, the Gm and the Inv locus, and Gm and $Gm^b$ have generally been considered to be alleles in the classical sense in that all human sera contain either $Gm^a$ or $Gm^b$ or both human sera lacking both Gm and $Gm^b$ have not been encountered (214) The recently discovered $Gm^{b2}$ (169) or $Gm^f$ (140) has caused some uncertainty as to the allelism in this system (81) The Gm and $Gm^b$ factors occur in the H chain of $\gamma$G globulin, are located on the Fc fragment obtained by papain digestion and are destroyed by pepsin (64 87) Gm and $Gm^b$ do not occur on the same $\gamma$G molecule. The Inv determinants are associated with the L chains and are found in all classes of immunoglobulin (64 87) $Gm^{b2}$ or $Gm^f$ is located on the H chain close to the disulfide bond holding the H and L chains together (169) The distribution among rabbit allotypes of the various determinants is quite similar in that *some are associated with* the L chains and some with the H and L chains close to the region

of the disulfide bond linking them. In the rabbit no determinants associated with the Fc fragment have yet been found. Finger printing of the Fc fragment and H chains indicates that a single peptide is found in Gm (a+) which is absent in Gm (a-) and a second peptide is present in Gm (b+) and Gm (f+) which is absent in Gm (b-f-) (67)

In this system of allotypic determinants once again a remarkable similarity between the myeloma globulins and antibodies emerges. Studies of myeloma γG globulins (88) have shown them all to contain but a single Gm or a single Inv factor indicating a monoclonal origin e.g. all myeloma globulins studied turned out to be Gm (a+) or Gm (b+) or Gm (a-b-) but none were Gm (a+b+). The existence of a considerable proportion of myeloma globulins of the class Gm (a-b-) shows that some clones of cells from Gm (a+), Gm (b+) or Gm (a+b+) individuals synthesize γG globulins without these determinants. Many of the Gm (a-b-) myeloma globulins possess $Gm^f$ ($Gm^{b-}$) determinants and KUNKEL *et al.* (119) have reported an association of the H chain subgroups with certain Gm patterns. Thus their Vi subgroup (TERRY and FAHEY $\gamma_2$C (219)) contained individuals with Gm (a-b-f-) myeloma proteins while their We subgroup ($\gamma_2$b) contained Gm (a+b-f-) and Gm (a-b-f+) myeloma globulins. Gm (a-b-f-) myeloma globulins occurred in all subgroups. The nature of this association of Gm factors and γG subgroups deserves further study since it offers the possibility of correlating at least two of the heterogeneities of γG globulins. The Inv factors of myeloma globulins behave identically to the Gm factors. Inv (a-b-) myeloma proteins are also quite common although as assayed on whole globulin Inv (a-b-) individuals are not found (214). $Inv^a$ determinants appear to be associated with type K and not with type L myeloma globulins and Bence Jones protein (64, 8 ). $Inv^b$ occurs only on K myeloma and Bence Jones proteins (88, 125)

Just as the study of myeloma γG globulins showed the presence of cells producing Gm (a-b-) γG globulin, so the study of antibody produced in various individuals showed that antibody γG globulin could lack the Gm and $Gm^b$ determinants. The absence of allotypic determinants on antibody γG globulin present in whole γG globulins was first shown in rabbits by GELL and KELUS (69) and by others (187) and has since been shown in other species. In man it was first recognized that all anti-D sera from

Gm or $Gm^b$ individuals were not suitable for coating erythrocytes for detecting $Gm^a$ and $Gm^b$ respectively (cf 214) in terms of our present knowledge most of these were almost certainly Gm (a–) and Gm (b–) proteins. When studies were carried out on purified human antibodies it was found that many antibodies lacked Gm or Inv determinants present on the whole γG globulin of the sera from which they were obtained. Thus in a study (2) of five anti bodies obtained from one individual whose whole γG globulin was Gm (a+b+) three of the antibodies, the antidextran, antilevan and antiteichoic acid of *S. aureus* were Gm (a–b–) a fourth, anti A, was Gm (a+b–) and the fifth, anti-tetanus toxoid, was Gm (a+b+) Since the Gm and $Gm^b$ titers of the whole γG globulin from this individual were much higher than the Gm and $Gm^b$ titers of the two purified antibodies which showed these Gm activities, the anti A was most probably a mixture of Gm (a+b–) and Gm (a–b–) molecules Similarly the anti-tetanus toxoid probably contained Gm (a+b–) Gm (a–b+) and Gm (a–b–) molecules. The antilevan from this subject was subsequently shown to be Gm (f+) but once again the titer was much lower than would be expected (131) The data are best interpreted on the hypothesis that contact with antigen causes chance stimulation of certain lines of cells which then elaborate antibody γG globulin with Gm determinants corresponding to those of the type for which their synthetic capacities were originally programmed. This is clearly substantiated since when different individuals form antibody to the same antigen viz antidextran, the antibody may be of any one or a combination of Gm groups regardless of the Gm group of the whole γG globulin save that an antibody possessing a Gm determinant not expressed on the whole γG globulin of the same individual has not been found (2)

Studies on the amino acid composition of purified antibodies were first carried out by KOSHLAND (116, 117) who showed that significant analytical differences in a number of amino acids occurred among three different antibodies, one to a positively charged (ammonium) one to a negatively charged (arsonic acid) and one to a neutral sugar (Lac) determinant antibodies to two of the anti gens were formed in single rabbits. Results in Table I show the amino acids in which differences were found. The anti-Lac antibody had about 5 tyrosines and 5 serine residues less and 7 aspartic acid residues more than the anti-arsonic acid. The latter had two argi

*Table 1*

Differences in amino acid composition of antibodies to various antigens formed in single rabbits

| | Residues per 100,000 g | | | |
|---|---|---|---|---|
| | Anti *Lac* (Rabbit 1) | Anti *Arsanil* (Rabbit 1) | (Rabbit 2) | Anti *Ammonium* (Rabbit 2) |
| Arg | 44.6 | 44.6 | 44.7 | 42.5 |
| Asp | 112 | 105 | 106 | 110 |
| Serine | 143 | 148 | 151 | 151 |
| Gly | 121 | 122 | 125 | 127 |
| Ala | 77.3 | 79.8 | 81.1 | 81.4 |
| Ileu | 47.2 | 48.1 | 48.4 | 46.4 |
| Leu | 89 | 89 | 89 | 91 |
| Tyr | 50.9 | 56.3 | 56.1 | 56.2 |

*From (116, 117)*

nines more and 4 aspartic acids, 2 iso-leucine and 2 glutamic acids less than the anti-ammonium antibody formed in the same rabbit. These differences did not appear to be related to the allotypes. Interpretation of these data in relation to the antibody combining site is made difficult by the occurrence of two populations of rabbit γG globulin molecules separable on DEAE Sephadex, which differ in the Fab fragment which they contain (83 164 208, 215) Antisera to various antigens and even to a single antigen (29) varied in the fraction with which they were associated. The chromatographic differences reported earlier (96) and the electrophoretic differences found (7) in the distribution of antibodies to different antigens, while not reinvestigated in terms of more recent findings, also present an additional difficulty in relating the findings to the antibody combining site. Allotypically different homozygous rabbits showed differences in amino acid composition (183)

In a study (12) of the amino acid composition of antibodies to four antigens, dextran, levan, teichoic acid and blood group A substance, formed in a single individual, even greater differences were found. Three of the antibodies were Gm (a−b−) even though whole γG globulin was Gm (a+b+) (2) The major differences in amino acid composition found are summarized in Table 2 The individual antibodies showed differences from one another amounting to as much as 32 valines, 20 glycines, 7 leucines and 7 prolines, 11 tyrosines, 12 arginines and 14 lysines. The sum of lysine plus arginine was almost constant (range 135 to 141 residues) for all four antibodies and for normal γG globulin from this individual. Two of

*Table II*

Significant differences in average amino acid composition among γG antibodies from one individual

| Gm Group | Antilevan a—b— | Antidextran a—b— | Antiteichoic acid a—b— | Anti-A +b— | Normal G a+b+ | Pooled M |
|---|---|---|---|---|---|---|
| Amino acid | | | | | | |
| Glycine | 101 | 99.5 | 120 | 108 | 107 | 105 |
| Valine | 132 | 134 | 102 | 115 | 139 | 105 |
| Leucine | 116 | 109 | 110 | 115 | 116 | 98.0 |
| Tyrosine | 45.5 | 56 0 | 53.3 | 53.2 | 55.9 | 41.0 |
| Arginine | 46.4 | 48.2 | 58.6 | 56.1 | 47.8 | 55.5 |
| Lysine | 93.5 | 87.3 | 81.9 | 79.1 | 93.2 | 64.0 |
| Hydroxylysine | 0 | 3.2 | 3.7 | 0 | 0 | 4.0 |
| Threonine | 113 | 109 | 110 | 127 | 111 | 116 |
| Proline | 101 | 101 | 108 | 108 | 107 | 97 1 |
| Arginine+ Lysine | 140 | 136 | 141 | 135 | 141 | 120 |

All values taken at 22 hours hydrolysis. Residues per mw 160,000.
From (12)

the antibodies contained 3 and 4 residues of hydroxylysine this amino acid was not found in the other two antibodies nor could it be detected in the whole γG globulin. Many of these differences may well have nothing to do with the antibody specificity since each antibody shows several different L chain bands on starch gel electrophoresis. The antidextran and anti-A contained both types K and L determinants while the anti-levan had only type K determinants. Since both fingerprint and amino acid analyses (32, 179 228) on types K and L Bence Jones proteins show very extensive differences not only between the two types but also among individual proteins of a given type, it is probable that many of the differences in amino acid composition are related to these factors. The amino acid composition of homogeneous antibody molecules remains unknown.

Recent studies by MILSTEIN (146) indicate the following C terminal amino acid sequence for types K and L Bence Jones proteins based on performic acid oxidation and on reduction and carboxymethylation.

Type I –Lys Ser Phe AspNH$_2$ Arg Gly Glu Cys
Type II –Lys Thr Val Ala Pro Thr Gly Cys Ser

and Gly Glu Cys has been isolated independently (180 146, 221) and is considered to be the C terminal peptide. In type K the C terminal cysteine is thought by MILSTEIN to be the link between the

L and H chains in γG globulin while in type II there is a C terminal serine and the subterminal cysteine forms the disulfide bond to the H chain. The C terminal peptides of types K and L from γG globulin occurred in about the same ratio as do the H and L chains themselves. It is not clear whether these sequences hold for all of the γG globulin molecules since human γG globulin has been reported to yield only glycine and serine by one group of investigators (126) and 2 moles of C terminal glycine and one mol each of C terminal serine and arginine by another (14) both groups used hydrazinolysis which destroys cysteine. Performic acid may also have an anomalous effect since it yielded a peak in the position of cysteic acid with insulin which does not contain C terminal cysteine (11) Discrepancies also exist with regard to the findings of the C terminal amino acids of rabbit γG globulin (14 210) While there appears to be a general impression that Bence Jones proteins and L chains are identical (cf 205) this may not be entirely true (147 213)

Substantial differences in amino acid composition of types K and L Bence Jones proteins have been reported (32 179 205 213 228) Initial amino acid sequence studies on individual Bence Jones proteins of type K show that they appear to have a uniform C terminal sequence but vary in the N-terminal portion of the molecule and myeloma globulins show individual antigenic specificity associated with the L chains and the Fd fragment of the heavy chain e.g in the Fab fragment (82) It is also of especial significance that the two types (K and L) of Bence Jones protein have no tryptic peptides in common (179) Such sequence studies on representative Bence Jones proteins of each type should ultimately reveal the nature of the structural variations of individual L chains (213) and in this manner contribute decisively to our understanding of the structural heterogeneity of gamma G globulin.

The high degree of individuality of different human antibody γG globulins is indicated by the production of specific antisera to antibodies which fail to react with antibodies to other antigens or with whole γG globulin from the same individual, or with antibodies to a given antigen formed by many other persons (120) Similar findings have been made in rabbits (161)

At present there is little or no evidence that any of the above heterogeneities involve the antibody combining sites themselves, although the existence of a system so complex that thousands or

more of distinct kinds of immunoglobulins are being synthesized continuously must have some far reaching implications with respect to antibody synthesis. Studies by Bernier and Cebra and Buffe *et al* by the fluorescent antibody technic indicate that only one type of H and one type of L chain are made in a given cell type K and L light chains were rarely if ever found in a single cell (17 23) While Pernis and Chiappino (167) confirmed this for red pulp of the spleen they found single cells in the germinal centers of splenic follicles and lymph nodes to produce both K and L chains. Some studies (155) but not others (200) on single cells from the rabbit showed that a considerable number could produce both γM and γG antibodies. These cells, in accordance with present concepts, would be producing two kinds of H chains although they might be producing only a single kind of L chain. Further studies on the synthetic capacities of individual cells with respect to producing more than a single H and a single L chain are of crucial importance. Since studies in several species have shown that animals and man frequently produce γM antibodies first followed by γG antibodies (13 15 52 80 224) it is of primary interest to confirm findings (155) that this can occur in single cells. Studies on single cells have also shown that individual antibody forming cells may produce antibody to two different antigens (8 153 154) and recently a considerable proportion of antibody forming cells (134) have been found to synthesize antibody to both the 9 and 12 antigenic determinants of *Salmonella* as assayed by immune adherence with bacteria containing only one or the other determinant. Similarly in studies (133) of individual cells from rabbits immunized with phage T6, the ratios of cross reactivity with T2 and T4 varied from cell to cell indicating that individual cells vary in the cross reacting potentialities of the antibody which they produce. A study by Attardi *et al* (9) showed that individual cells varied in the kinds of phage neutralizing antibody which they produced, 19 of 29 antibody forming cells producing an antiphage which neutralized T5 phage growing on cells of *E. coli* of strains B and F while the remaining cells produced antibody neutralizing phage growing only on either one of the two strains but not on the other

Thus while the studies of myeloma proteins indicate production of a single homogeneous immunoglobulin by each type of

This could, however be antibody of 9 12 specificity and not two antibodies with 9 or with 12 specificity

myeloma cell the findings on antibody formation by single cells have not tended to support the idea that homogeneous populations of antibody molecules would result even if single cell lines possessing the capacity to synthesize antibody could be obtained. There is no evidence indicating whether or not the neoplastic change has any restrictive effects on the capacities of cells to synthesize proteins as compared with normal cells. It will be of decisive importance to theories of antibody formation to evaluate the range of potentialities of individual cells with respect to the synthesis of various immunoglobulins, of antibody to different antigens, antigenic determinants and indeed, to synthesize the heterogeneous population *of antibody molecules formed even to a single antigenic determinant.*

Having surveyed the parameters of heterogeneity with respect to the compositional, antigenic and physicochemical properties of the immunoglobulins, there remains to consider heterogeneity with respect to the antibody combining site itself This type of heterogeneity arises from two main features. The first of these is the complexity even of single purified antigenic macromolecules in that they generally have multiple antigenic determinants and the second is that even toward a single antigenic determinant the antibody formed is not homogeneous, but consists of populations of antibody molecules with combining sites of various sizes or directed toward different aspects of an antigenic determinant. There are also a variety of subsidiary types of heterogeneity which are or probably will eventually be explainable in terms of these two main features. Among these are 1) cross reactions due to similarities in structure between one or more of the antigenic determinants of various polysaccharide or protein antigens 2) the existence of precipitating and non precipitating antibodies 3) the existence of antibodies giving the flocculation as distinct from the precipitin type of curve (cf *106*, *148*) A number of other variations (cf *236*) may be attributable to combinations of factors involving physical or chemical properties of the individual antibody molecules as well as those involving the antibody combining sites themselves.

Efforts to degrade protein antigens to obtain fragments possessing one or another of the antigenic determinants of the intact molecule and using modern immunochemical methods for characterizing the fragments were first carried out on human albumin using rabbit spleen extract by LAPRESLE *et al* (*123*) and on bovine albumin using cathepsin by PRESS and PORTER (*177*) and have

subsequently been extended to many other protein antigens (cf 182, 185 106, 123 for additional references) In general, digestion of an antigen produces fragments each of which may contain several of the different antigenic determinants present in the intact molecule in that each fragment reacts only with a portion of the total antibody to the intact antigen. Thus, partial digestion of human albumin yielded three fragments each of which formed a distinct band in agar diffusion with an antiserum to human albumin all three of the bands fused completely with that formed by the native albumin. On further digestion precipitating power was lost but fragments retained the capacity to combine with antigen to inhibit precipitation. This inhibitory fragment had a molecular weight of about 11 000 and was bivalent (123) By treatment with trypsin a smaller fragment of molecular weight 6,600 was isolated which had only a single determinant group (124) This fragment when coupled to an insoluble adsorbent (p-aminobenzyl cellulose) removed only one per cent of the total antibody from antisera to human albumin. The antibodies to this univalent fragment eluted from the adsorbent reacted specifically to form a complex containing two molecules of inhibitor per molecule of antibody indicating their bivalence the bond between the fragment and the antibody combining site was so firm that free fragment and antibody were not observed (124) The finding that this inhibitory fragment containing a single determinant reacts only with so small a portion of the total anti-serum albumin indicates the high degree of heterogeneity of the antibody response to a protein antigen. While ultimately fragments containing antigenic determinants may be obtained toward which a much larger proportion of the total antibody is directed, it is evident that in the immediate future, except by extremely good fortune, antibodies to individual determinants of protein antigens will not be available in large amounts. Establishment of the number of distinct antigenic determinants of protein antigens and their isolation in purified form represents a major direction for further immunochemical research. Since a bivalent fragment has been shown not to precipitate with antibody (123) and since the three fragments obtained on digestion of albumin each precipitate, one has at present no estimate of the number of different determinants on a protein antigen.

To obtain substantial amounts of antibody toward a single antigenic determinant one must turn to homopolysaccharide anti

gens (cf 105–107) to polypeptide antigens of simple structure (141 207) and to antigens containing determinants of known chemical structure (5 196 199) which have been introduced on to proteins polypeptides etc. Each of these procedures has certain advantages and none are without their limitations. All of the studies to date indicate clearly that even toward a single antigenic determinant the antibody formed is not homogeneous.

After it was demonstrated that dextran was antigenic in man, studies on the dextran-human antidextran system provided the first indications of the size and heterogeneity of the antibody combining sites to a single antigenic determinant (cf 105 107) By assays of the capacities of oligosaccharides of the isomaltose series to inhibit precipitation of antidextran by dextran it was found that the increment in inhibiting power decreased with chain length reaching an upper limit since, with most human antidextrans, isomaltohexaose and isomaltoheptaose were equal in potency on a molar basis in one instance the heptasaccharide was somewhat more potent than the hexasaccharide. The dimensions of isomaltohexaose in its most extended form were 34×12×7 Å and its molecular weight was 990. Subsequent studies with other polysaccharide (cf 105–107) and polypeptide determinants for homologous and cross reactions have given comparable values for the size of the units fitting into antibody combining sites (5 25 130 141 196)

That the antidextran formed to the α1 6 linked glucose determinants of dextran was not a homogeneous population of molecules but consisted of mixtures of antibody molecules with combining sites of various sizes, as indicated by their varying affinities for smaller as compared with larger oligosaccharides of the isomaltose series, *was first inferred by examining the inhibition curves for the* antidextrans formed indifferent individuals (cf 105–107) Fig 1 shows that the ratio of inhibiting power relative to isomaltohexaose of the smaller oligosaccharides varied from one antiserum to an other a behavior which would not be expected were all antibody molecules homogeneous with respect to the sizes of their combining sites. In the SIII rabbit anti-SIII system similar heterogeneity was inferred from differences in the inhibiting capacity of SIII oligosaccharides for various antisera (132) Heterogeneity has also been found by equilibrium dialysis measurements with rabbit antisera to *p*-nitrophenyllactoside and indicates differences in binding affinity for various portions of this determinant (110)

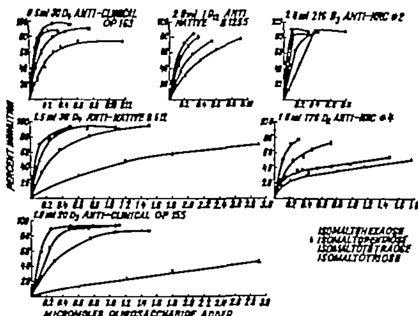


Fig 1 Comparison of inhibiting power of isomaltotriose, -tetraose -pentaose and -hexaose with six human antidextran sera of 1,6 specificity From (106)

It was possible to obtain direct evidence for heterogeneity involving sizes of the antidextran combining sites by obtaining a partial fractionation of the antidextran from the serum of a single individual into two or more subfractions which differed in the relative inhibitory capacities of smaller and larger oligosaccharides to one another (70 71 198) These subfractions also differed in the amounts needed to give comparable complement fixation with dextran. The principle involved was to absorb the antidextran on an insoluble dextran (Sephadex) and to elute sequentially from the thoroughly washed precipitate with oligosaccharides of increasing chain length. In earlier studies elution with isomaltose (IM2) or isomaltotriose (IM3) was used first to extract a portion of the antibody and the remaining antibody was subsequently extracted with isomaltohexaose (IM6) More recently (71) washed Sephadex antidextran precipitates have been extracted sequentially with glucose, methyl-α-D-glucoside, IM2, IM3 IM4 etc.

Results are shown in Fig 2. It is evident that the antibody eluted by IM3 has a lower capacity to give fixation of complement

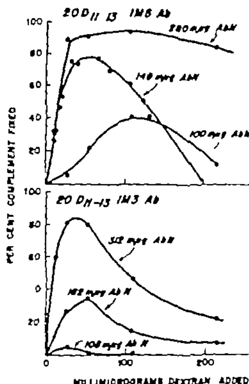



*Fig. 2.* Comparison by quantitative complement fixation tests of fractions of human antidextran. – Lower graph: Antibody extracted from washed Sephadex-antidextran precipitate by isomaltotriose. Upper graph: Antibody subsequently eluted by isomaltohexaose. From (70)

in the presence of dextran than does the antibody subsequently eluted by IM6 e.g. significant fixation was obtained with as little as 100 ng AbN of the latter while negligible C' fixation was obtained with 108 ng of the former. Similarly 140 ng N of the IM6 Ab gave about 80% fixation while the 182 ng N of the IM3 Ab gave only about 35%. When maximum fixation was obtained the IM3 Ab was more readily inhibited in antigen excess.

The studies on inhibition of precipitation (198) and of C' fixation (70) by the IM3 Ab and the IM6 Ab are shown in Fig. 3. It is evident that IM2 and IM3 give considerable inhibition with the IM3 Ab extract while giving little or no inhibition with the IM6 Ab. Similarly the differences in inhibiting power of IM5, 6 and 7 are greater for the IM6 Ab than for the IM3 antibody. These findings strongly substantiate the hypothesis that the antidextran in the

whole serum is a heterogeneous population of antibody molecules with combining sites which differ in the dimensions of their complementary regions.

Sequential elution (71) with IM2, IM3 IM4 and IM5 have given further indications that some fractionation with respect to the sizes of the complementary areas on the antibody molecule may be achieved. Fig 4 shows the properties of the antidextran obtained by such sequential elution. It is evident that the relative inhibiting power of IM2 to IM7 is less in the IM2 than in the IM3 eluate which in turn is less than that in the IM4 eluate in the IM5 eluate, IM2 was so poor an inhibitor that the 50% end point could not be determined. Table III summarizes the relative inhibiting power of the various oligosaccharides in the 4 eluates. It is seen that for all 4 eluates the relative inhibiting powers of IM5 and IM6 relative to IM7 is constant and also for the three eluates in which it was measured that the relative inhibiting power of glucose relative to IM7 was constant. However with oligosaccharides from methyl α-D-glucoside to IM4 there is a progressive decrease in inhibiting

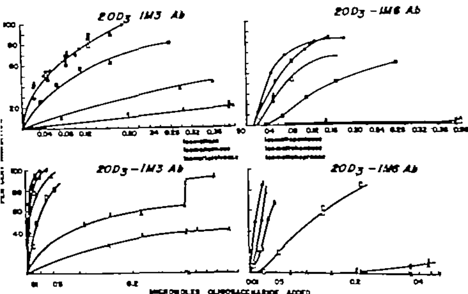



*Fig* 3. Inhibition by isomaltose oligosaccharides of precipitation (upper graphs) and complement fixation (lower graphs) of human antidextran fractions by dextran.
From (70)

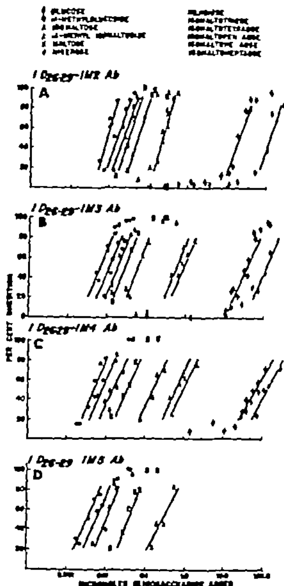



*Fig 4* Inhibition by oligosaccharides of complement fixation with dextran of purified human antidextran fractions obtained by sequential elution of washed Sephadex-antidextran precipitate with isomaltose, isomaltotriose isomaltotetraose and isomaltopentaose From (71)

power of the oligosaccharide relative to IM7 with the antidextran eluted by the higher oligosaccharides. Thus with this antiserum the bulk of the heterogeneity in sizes relates to the areas of the determinant from the first α-glycoside linkage through the fourth

glucose residue. With another antiserum evidence for heterogeneity involving the fifth glucose residue was obtained (71) Heterogeneity involving over a 10 000 fold variation in intrinsic association constant was found on fractionation of individual rabbit antisera to DNP antibodies (44)

Initial attempts at refractionating the eluates by reabsorption on Sephadex and again eluting with oligosaccharides have not been very successful in affecting further fractionations of the antibody and losses in processing especially due to formation of insoluble materials during ultrafiltration were considerable. In further studies in collaboration with Dr MARIANNE N DORNER, separation of oligosaccharide from the antibody has been effected much more readily on columns of Biogel P10 with greatly improved yields and no formation of insoluble materials. This makes possible much more extensive fractionation. One possible difficulty in obtaining homogeneous antibodies by this subfractionation procedure would be if both antibody combining sites on a single antibody molecule were not of the same size or binding affinity If a considerable proportion of the antibody molecules possessed one combining site directed toward a larger oligosaccharide (e.g IM6) and one toward a smaller (e.g IM2 or IM3) such antibody would distribute itself in all fractions. Difficulties in subfractionation may in part be due to such a circumstance (cf 71)

The use of insoluble adsorbents has also been extended to other antibodies. Dr B. INGELMAN of Pharmacia Co. has prepared a sephadex type material from levan and this has been used successfully to remove antilevan. In the case of the blood group A and

*Table III*

Relative inhibiting power of various oligosaccharides with 1 D[illegible] purified antidextran fractions

| Fraction extracted with | IM7 | IM6 | IM5 | IM4 | IM3 | Methyl-α-IM2 | IM2 | Methyl-α-D-Glucoside | Glucose |
|---|---|---|---|---|---|---|---|---|---|
| IM2 | 100 | 59 | 38 | 29 | 17 | | 4 | 0·04 | 0·007 |
| IM3 | 100 | 59 | 36 | 23 | 11 | 1 2 | 0·77 | 0·018 | 0·0059 |
| IM4 | 100 | 65 | 33 | 14 | 3·3 | 0 84 | 0·53 | 0·008 | 0·0052 |
| IM5 | 100 | 60 | 35 | 9 | 1 2 | | ca. 0·2 | | |

Percentage inhibiting power at the 50 % inhibition point relative to IM7.
Eleven per cent inhibition, highest point tested (cf. Fig. 4D) was compared with equivalent value for IM7 by reading from IM7 line at 11 % inhibition.
From (71)

B substances, addition of polyleucyl residues to the free amino groups by use of N-carboxy-leucylanhydride has yielded insoluble antigens capable of absorbing anti A or anti B from specific anti sera (109) Antibody may be eluted with acid or preferably with specific monosaccharides, such as N acetylgalactosamine for anti-A and galactose for anti B and also by specific blood group oligosaccharides. An N-acetylgalactosamine eluate was found to contain γG γM and γA antibodies and these possessed K and L determinants. Further fractionation should yield the three classes of antibodies separately so that the binding affinities and heterogeneities of their antibody combining sites may be studied.

Heterogeneity directed toward different aspects or spatial configurations of antigenic determinants is evident especially from studies on the glycerol teichoic acid of the group A streptococcus (142) The teichoic acid is a chain of glycerol phosphates linked 1 ⟶ 3 with alanine esterified on carbon 2 of the glycerol. Antisera to whole streptococci may show several types of behavior with glycerol teichoic acid and its derivatives. One type of antibody reacts with the intact alanine containing polymer but not with the polymer after splitting off of the alanine. A second type of antibody does not react with the alanine containing polymer but reacts after removal of the alanine. The third type precipitates equally well with both antigens. Thus the specificity of the last type of antibody *must be directed against a conformation of the polyglycerol phosphate* in which neither the alanine nor the secondary hydroxyl enters into the antibody combining site.

In a similar manner the A B cross reacting antibody present in human sera containing anti-A and anti B appears to have a specificity for the terminal N-acetylgalactosamine but which does not involve the N acetyl group (197) Since the terminal non-reducing end of the blood group A determinant is an α-linked N acetylglucosaminyl residue while that of the B determinant is an α-galactosyl residue, an antibody formed to the portion of the residue not involving the substituent on carbon 2 would be expected to have the same specificity for both and hence exhibit the properties of the A B cross reacting antibody

In linear polysaccharides with more than a single sugar heterogeneity could also result from the antibody forming mechanism giving rise to combining sites differing in the sugar toward which the maximum binding energy is directed. Thus both in linear and

branched oligosaccharides the increment in inhibiting power of oligosaccharides of increasing size decreases with chain length (105–107 132) Maximum contribution to binding affinity is found in branched polysaccharides for the terminal non-reducing sugar unit (105–107) Were each of the different sugar residues on the linear polysaccharides to serve as the equivalent of the non-reducing end in influencing antibody complementarity two different heterogeneous populations of antibodies would be formed to the single chain (cf 107)

In the consideration of the properties of the heterogeneous array of proteins possessing antibody properties, one must consider also their valence. Overwhelming evidence by equilibrium dialysis, fluorescence quenching and by studies of the fragments obtained by peptic or papain digestion (cf 31 44 106 110 206) and by electron microscopy of antigen-antibody aggregates (3 51 121) indicates the bivalence of γG antibody Data are also being accumulated to indicate that the γM antibody has a valence of 5 or 6 (157 95) KLINMAN *et al.* (113 188) have found that equine antilactoside antibody of the γA type had a valence of two by equilibrium dialysis and also that it consisted of a heterogeneous population of molecules with combining sites of different binding affinity The affinity of the γA antibody was higher than that of the precipitable antibody from the same horse but it was non-precipitable. Thus non-precipitable antibodies are not univalent failure to precipitate may be related to the arrangement of the binding sites so that if one site is occupied by a large antigen molecule, it interferes sterically with interaction at the second site. Evidence that papain digestion of γA antibodies gives a divalent fragment which yields univalent fragments on reduction has also been obtained by WEIR (cited in 31) Study of the nature of the binding affinities of the various classes of antibodies, γA, γM and γG toward the same antigenic determinant is just beginning and will undoubtedly contribute substantially to our knowledge of antibody structure and heterogeneity

*Biological Properties of Antibody Associated with Heterogeneity*

Considering the extreme degree of heterogeneity of antibody molecules as outlined above it would be most extraordinary if this were not reflected in the biological activities of antibody Variation

in the protective power or neutralizing ability of various antisera, notably horse antitoxic sera, which were not attributable to variations in the amount of antibody were encountered as soon as such antisera were introduced for therapeutic purposes and necessitated *the introduction of rather complex and indirect methods of standardization* these variations are now understandable in terms of the heterogeneity of antibodies (cf 182, 185) In studies on antibody to ribonuclease antibodies inhibiting enzyme activity and those not inhibiting enzyme activity are found the latter block the action of the inhibiting antibody (28)

Similarly the capacity of antisera to induce systemic and passive cutaneous anaphylaxis and passive sensitization of intestinal or uterine muscle has been shown to vary in certain species. It is of especial interest that rabbit antibodies in which most of the earlier studies were done were more uniform in biological activity per microgram antibody N than were antisera of other species and as mentioned above, rabbit antisera were much less heterogeneous with respect to chemical and physical parameters. The antibodies in most hyperimmune sera were almost exclusively of the γG variety Thus, protective power of rabbit antipneumococcal sera was generally proportional to the anti-type specific polysaccharide as determined quantitatively and rabbit antibodies behaved relatively uniformly in their capacity to sensitize passively for systemic anaphylaxis, cutaneous anaphylaxis and *in vitro* sensitization of intestinal muscle this uniformity extended to antibodies to different antigens as well as to different antisera to the same antigen (cf 106, 162) It was only an apparent uniformity however since fractions of differing sensitizing power could be obtained from individual sera (158)

On the other hand when human antibodies and antibodies of *other species were used substantial variations in sensitizing capacity* were noted. Thus γG antibodies to picryl or DNP proteins formed in the guinea pig (20) and mouse (156) differed in their capacity to sensitize guinea pig skin, only the faster moving γG antibody giving passive cutaneous anaphylaxis. Similarly when human antibodies were studied, extraordinary differences in capacity to sensitize intestinal muscle were found for purified antidextrans as compared with diphtheria antitoxin in whole serum, the former being 1000–3000 times less potent even though it was in purified form and free of other non specific γG globulins *known to interfere with*

sensitization. Differences of one hundred fold were also found among human diphtheria antitoxic sera per $\mu g$ antitoxin N in their sensitizing capacity for intestinal muscle. The antidextran was also 25 to 100 times less potent in producing PCA than was the antitoxin (108) and individual antitoxic sera also varied in the quantities of antibody needed for PCA. These variations are probably very closely related to differences in the capacities of whole $\gamma G$ globulins of various species to inhibit sensitization for PCA in the guinea pig as well as to differences in their capacities to induce reverse passive sensitization. Which, if any of the chemical parameters of heterogeneity are responsible for these differences is not yet understood except that the capacity to attach to guinea pig skin of $\gamma G$ globulin from those species which sensitize is associated with the Fc fragment obtained on papain digestion and TERRY (218) has recently shown that only myeloma proteins of $\gamma G_1$ and $\gamma G$ subgroups can sensitize for reverse PCA while those of the $\gamma G$ subgroup did not. Both of these observations indicate that capacity to attach to guinea pig skin is associated with the H chain and specifically that portion of it in the Fc fragment.

To the extent that they have been studied $\gamma M$ and $\gamma A$ antibodies have not produced PCA in the guinea pig nor have normal $\gamma M$ and $\gamma A$ globulins nor 11 myeloma $\gamma A$ globulins of the K and L types been found to sensitize for reverse PCA (65) Antibodies with capacity to sensitize human skin for the PRAUSNITZ KÜSTNER (PK) reaction have now been shown to be of the $\gamma A$ and $\gamma G$ classes (53 54 72, 73 97–99 217 226) The inability of non precipitating antibodies to produce Arthus reactions as contrasted with precipitating antibody (cf 106) has been associated with failure of the non precipitating antibody to form an antibody-antigen host complement complex in blood vessel walls (129) However it reacts with antigen and produces passive cutaneous anaphylaxis as well as precipitating antibody does.

Another important biological property of certain antibodies is their ability to cross the placental barrier from mother to fetus (cf 21) Extensive studies on antibody levels in mother and fetus have been made in various species for many years (cf 31) More modern findings indicate that only $\gamma G$ and not $\gamma A$ or $\gamma M$ immunoglobulins of various species including man can traverse the placenta thus placental transmission is associated with the Fc fragment (cf 31) which has been shown to traverse the placenta more readily than

the Fab fragment. These findings also account for the failure of the γM saline anti Rh agglutinins to pass from mother to fetus while the γG blocking Rh antibodies traverse the placenta readily Human PRAUSNITZ KÜSTNER antibodies also have been reported not *to pass from mother to fetus (cf 106) In the rabbit* γM as well as γG molecules have been reported to pass into the fetal circulation. Both fast and slow moving guinea pig antibodies were found to cross the placenta (20)

Variations in the complement fixing capacity of antisera during the course of immunization have been reported even for rabbit antisera. Thus rabbit antisera to bovine serum albumin formed after one month of immunization had a lower capacity to fix complement per μg antibody N and showed much greater variation from animal to animal than did antisera formed after more prolonged immunization (231) Similar variation was encountered among rabbit antipneumococcal sera (93) and among fractions of antibody obtained from single rabbits. The injections schedules were such that both kinds of antibodies were almost certainly γG globulins. More recent studies in guinea pigs have shown that fast moving γG globulin of guinea pig anti DNP and anti-picryl antibodies which gave PCA could not fix C while the slower moving γG antibody fixed C (19 163) While some γM and γG antibodies fix C with homologous antigen other γM and γG antibodies do not and there are substantial species differences which are not fully understood (cf 31 106) Thus horse antipneumococcal antibody failed to fix C while cow antipneumococcal antibody does both are γM globulins (104) The few γA antibodies thus far studied did not fix (cf 31) γG globulin on aggregation by heat acquires C fixing properties (100) Mercaptoethanol (0 1 M) and iodoacetamide treated γG antibodies no longer fixed complement *although they reacted with antigen normal γG globulin treated* similarly lost its capacity to fix complement on aggregation (234) Rabbit and sheep γG antibodies could be shown to lose their complement fixing capacity upon reduction of about seven disulfide bonds but without loss of precipitating power or fragmentation of the molecule until more than 10–11 bonds were reduced. A single labile disulfide bond appears to be of importance for C' fixing capacity (202 204)

Although the Fc fragment possesses the power to fix C' on aggregation *by heat or by coupling with bisdiazobenzidine (100)* and

can react with C' to inhibit its subsequent reaction with sensitized sheep erythrocytes (4 216) C' fixation involves other portions of the molecule as well. Thus papain digestion of specific precipitates which had fixed C' yielded Fab fragments containing bound C' (184) and specific precipitates from antibodies which had been digested with pepsin to destroy Fc showed 40% of the C' fixing capacity of whole antibody (202 203) surprisingly formation of specific precipitates in the presence of guinea pig complement gave no fixation. Furthermore digestion of rabbit antibody with insoluble papain without reduction does not affect C' fixation on reduction the Fc fragment did not dissociate completely as it would have in the absence of C suggesting that the C' was bound to the Fc and Fab fragments (26)

It should be emphasized that since γG globulin solutions have been found to show degradation on standing (211) perhaps due to enzymatic action (209) some of the differences in biological activities may not be due to heterogeneity but to such degradative changes. Efforts to exclude such changes should be made on the preparation used. ε-Aminocaproic acid (212) and trypsin inhibitor are used to prevent such effects (cf 171)

### *Effect of Mercaptoethanol on Antibodies*

γM antibodies in whole serum are generally destroyed by treatment with 0 1 M mercaptoethanol as measured by hemagglutination and this procedure is widely used as a screening test for such antibodies (34 85) Under these conditions except for impaired capacity to fix C' as outlined above, γG antibodies retain their capacity to precipitate or hemagglutinate. γA isoagglutinins showed intermediate susceptibility to mercaptoethanol (99) It has been shown that the fragments produced from γM antibodies by mercaptoethanol retain capacity to bind antigen and on reassociation antibody molecules possessing precipitating or hemolytic activity are formed in appreciable amounts (101 102, 201) Accordingly these conditions of reduction probably do not cause disruption of the combining sites themselves but are causing fragmentation of the molecule with the formation of monovalent fragments.

### *Nature of the Antibody Site*

Several studies have been carried out in efforts to establish which of the chains of γG globulin are involved in the antibody

combining site. Most workers find that the H chains obtained by Sephadex chromatography in propionic acid of mercaptoethanol treated γG antibodies (55) retain a substantial part of the binding capacity for antigen of the intact molecule (cf 31) indeed in one study (225) up to 85% of the original binding antibody was present in the H chain which was estimated to contain less than 4–5% of L chain. The isolated L chains from antibody γG globulin are generally conceded to be completely inactive. However studies from several laboratories (43 56 57 190) have indicated that recombination of H and L chains yields greater antibody activity than was present in the isolated H chains alone some increase in antibody activity also resulted from addition of non-specific L chains to H chains derived from antibody With horse diphtheria antitoxin and with cow and pig antibody to the dinitrophenyl group however isolated H and L chains formed by S-sulfonation both have been found to be inactive but they could be recombined with restoration of antibody activity (56, 57 57a) activity of H chains reported by others was attributed to contamination with higher molecular weight materials. The anti DNP activity recovered was 40% of that of the original antibody or 80% of that of the S-sulfonated chains before they were separated. L chains of non-specific origin also restored activity but to a lesser degree than specific L chains. Studies on the H chains of normal γG globulin and L chains of Bence Jones protein have shown that they can reassociate to form intact γG globulin (68) and that if recombination occurs in the presence of antigen, antibody L and H chains recombined preferentially (144) Additional evidence for involvement of both the H and L chains in the antibody combining sites comes from attempts to label the antibody combining site specifically (145) or to prevent its labelling by blocking the site with hapten (190) Both types of study indicated that H and L chains are involved. At present further data are needed to evaluate the contribution of the L chains it may well be that they exert a stabilizing effect on the site.

Unfolding the Fab fragment in concentrated guanidine HCl as measured by optical rotation studies and subsequent renaturation by dialysis to remove the reagent yield a considerable portion of the original activity (22 152) Similarly denaturation of the Fab fragment of rabbit antiribonuclease with 8 M urea and 0 1 M mercaptoethanol removal of the urea by dialysis and oxidation of

the SH groups by air restored a considerable portion of antibody binding activity (86) While these studies are very exciting and open new areas for study questions remain as to the completeness of disruption of the combining site itself which represents but a small portion even of the Fab fragment.

The heterogeneity of antibodies thus appears to be the unique characteristic of the immunoglobulins and an understanding of the ultimate basis for this remarkable phenomenon may be the *sine qua non* for solving the problem of antibody synthesis.

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# Les études quantitatives de l'isohémagglutination

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Nous avons cherché à rassembler dans ce rapport les principaux résultats d'intérêt biologique qui ont été obtenus par l'étude quantitative des isohémagglutinines humaines du système ABO.

Depuis la découverte de LANDSTEINER les recherches sur l'isohémagglutination ont surtout concerné la chimie des substances des groupes sanguins et les règles de leur hérédité. Les connaissances sur les anticorps correspondants sont restées longtemps plus limitées. Leur étude est entrée dans une phase nouvelle, quand une méthode quantitative y a été introduite. Cette méthode consiste à appliquer la thermodynamique à la fixation des agglutinines sur les hématies. Les données que l'on obtient ainsi apportent des informations sur l'énergie, la nature, le nombre des liaisons mises en jeu dans l'union de l'anticorps à l'antigène. Elles peuvent donc servir à l'étude physicochimique du phénomène d'agglutination. Mais ces données sont aussi les éléments d'une caractérisation des structures qu'aucune autre méthode ne peut fournir actuellement et qui a permis de connaître des faits nouveaux dans le domaine de la génétique chimique. C'est le seul aspect de la question qui sera traité ici.

## *La méthode thermodynamique*

Il était nécessaire, pour appliquer la thermodynamique à la première phase de l'agglutination, de disposer d'un procédé de dosage des agglutinines et de s'assurer que leur fixation était réversible. La technique mise au point par FILITTI WURMSER et ARMAND JACQUOT (1) repose sur le fait qu'à une température $\theta$ donnée, et dans des conditions définies, une quantité déterminée d'une certaine espèce d'agglutinine est capable de provoquer l'agglutination d'un nombre maximum déterminé d'hématies. Il a été

montré (1 2) que le nombre maximum $N_4$ obtenu à 4°C peut être pris comme une mesure relative de la concentration de l agglutinine

FILITTI WURMSER et JACQUOT ARMAND (1) ayant démontré en outre la réversibilité du phénomène de l isohémagglutination, il a été possible de vérifier l application de la loi d action de masse à la combinaison des agglutinines avec l agglutinogène – FILITTI WURMSER, JACQUOT ARMAND et WURMSER (3) – Pour des raisons géométriques, l agglutinine, même si elle possède deux groupes ré actifs, se comporte comme si elle était unifonctionnelle. La théorie est alors très simple. Considérons une hématie H comme une très grande molécule, possédant $n$ groupes agglutinogènes identiques, et indépendants les uns des autres, chacun étant capable de se combiner avec une molécule de l agglutinine A. Il existe dans le système en équilibre, selon la concentration de A, un plus ou moins grand nombre d'hématies H ayant combiné 1 2, 3 $n$ de leurs groupes. Bien que nous ayons supposé que tous les groupes sont exactement de même nature, nous pouvons les distinguer par leurs positions sur l hématie H. Considérons alors l équilibre régnant entre A et deux sortes d'hématies, celles qui ont un certain groupe spécifié vacant, et celles qui ne diffèrent des premières que parce que ce groupe est combiné avec A. La première sorte sera désignée par $HA_{s,i}$ où $s$ est le nombre de molécules d agglutinine fixées et $i$ sert à désigner la distribution particulière des $s+1$ molécules sur l'hématie. L équilibre est donc représenté par l équation



$$HA_{s,i} + A \rightleftarrows HA_{s+1,i}$$

On peut définir une constante intrinsèque K caractéristique des groupes spécifiques de l antigène et de l anticorps

$$K = \frac{(HA_{s+1,i})}{(HA_{s,i})\,(A)}$$

(A) étant la concentration d agglutinine restée libre, et les autres termes entre parenthèses représentant les concentrations respec tives des deux sortes d'hématies une fois l'équilibre établi.

Une fois l équilibre atteint dans un mélange de $N_i$ hématies avec un sérum dont la concentration initiale en agglutinine était $N_{4i}$, on pourra connaître la concentration (A) de l agglutinine restée libre en centrifugeant le mélange et en mesurant le nombre maximum $N_4$ des hématies qui peuvent être agglutinées par le surna

geant. La différence $N_f = N_i - N_4$ est proportionnelle à l'agglutinine fixée. Appelons $\varphi$ le coefficient d'équivalence entre le nombre $N_4$ et la molarité de l'agglutinine. En considérant que dans 1 mm³ d'une solution molaire d'hématies il existerait $6 \times 10^{17}$ hématies, on peut montrer que l'on doit avoir d'après la loi d'action de masse

$$\frac{N_i}{N_f} = \frac{6 \times 10^{17}}{n\,\varphi} + \frac{6 \times 10^{17}}{n\,K\,N_4} \qquad (1)$$

Si l'on porte en ordonnées $N_i/N_f$ et en abscisses $1/N_4$ on obtiendra une droite dont la pente sera inversement proportionnelle à $nK$. Puisque $n$ est le nombre de groupes agglutinogènes présents sur une hématie et est pratiquement une constante, la pente sera d'autant plus abrupte que l'affinité de l'agglutinine pour un même agglutinogène sera plus faible.

L'isoagglutinine anti B a été étudiée sur 61 sérums, soit 36 d'individus $A_1O$, 8 d'individus $A_1A_1$, 5 du sous-groupe $A_2$ et 12 du groupe O.

Un résultat qui s'est dégagé de nos recherches est que les droites obtenues avec tous les sérums normaux provenant d'individus d'un même génotype ont la même pente et que cette pente varie avec le génotype.

Nous avons appelé $\beta(0)$, $\beta(A_1A_1)$, $\beta(A_1O)$, $\beta(A_2O)$ les agglutinines anti B présentes dans ces sérums. Les recherches *ont ultérieurement* porté sur les agglutinines $\alpha$ et $\alpha_1$ de 14 sérums d'individus B0 et 9 sérums du groupe 0

*Tableau I* (3, 4, 5, 6)

| | $(N_i / N_f)$ [illegible] | | | |
|---|---|---|---|---|
| | [illegible] | $\beta(A_1A_1)$ | $\beta(A_1O)$ | $\beta(A_2)$ |
| 37° C | 4,0±0,14 | 2,3±0,08 | 1,5±0,07 | 0,43±0,07 |
| 25 C | 3,7±0,04 | 1,5±0,07 | 0,5±0,08 | 0,25±0,04 |

*Tableau II* (2, 7, 8)

| | $(N_i / N_f)$ [illegible] | | | | |
|---|---|---|---|---|---|
| | Sérums du groupe B | | | Sérums du groupe 0 | |
| | $+ A_1$ | $+ A_2$ | $\alpha_1 + A_1$ | $+ A_2$ | $\alpha_1 + A_1$ |
| 37° C | 2,5±0,25 | 12,4±0,6 | 100±2 | 7,2±0,3 | 18,6±0 45 |
| 25 C | 0,7±0,07 | 3,3±0,2 | 13±0,16 | 5,1±0,2 | 10,5±0,6 |

On doit à SALMON (10 11) une étude de l anticorps anti B du phénotype exceptionnel Ax. Les pentes à 37 et 25 C sont respectivement 2 6 et 1 04

Quand, ayant tracé les droites représentant $(N_l/N_f)$ en fonction de $(1/N_4)$ à une certaine température, on trace les droites analogues pour une autre température, on observe que la pente, et par conséquent la constante intrinsèque d association K, varie avec la température d une manière différente pour les sérums de génotypes différents. En appliquant la relation de VAN'T HOFF on calcule les chaleurs de réaction (−ΔH) correspondant à la combinaison d un groupe agglutinogène avec une molécule d agglutinine. Le tableau III indique les données ainsi établies dans notre laboratoire pour les groupes sanguins classiques du système ABO et pour le Ax étudié par SALMON

*Tableau III*

| | | −ΔH | kcal/mole |
|---|---|---|---|
| $\beta(A_1O)$ | + hématies B | 16 | ± 2 |
| $\beta(A_1O)$ | + stromas B | 16 | |
| $\beta(A_2A_1)$ | + hématies B | 6,5 | ± 1 |
| $\beta(A_2O)$ | + hématies B | 9 | |
| $\beta(OO)$ | + hématies B | 1 7 | ± 0,4 |
| $\beta(Ax)$ | + hématies B | 14 | |
| $\alpha(B)$ | + hématies $A_1$ | 19 | ± 3 |
| $\alpha(B)$ | + hématies $A_2$ | 20 | |
| $\alpha_1(B)$ | + hématies $A_1$ | 31 | ± 0,5 |
| $\alpha_1(OO)$ | + hématies $A_2$ | 8,7 | |
| $\alpha(OO)$ | + hématies $A_1$ | 5,3 | |

*Le rapport $N_4/N_{37}$*

La méthode thermodynamique exige la détermination de l agglutinine restée libre. Or il est possible de mettre en évidence plus simplement, mais de manière très approchée, la plus ou moins grande exothermicité de la combinaison de l agglutinine avec l'hématie. La mesure des nombres maximum $N_4$ et $N_{37}$ d'hématies agglutinées aux deux températures 4 C et 37 C, permet en effet d obtenir un classement des sérums qui jusqu ici s'est montré le même que celui obtenu par la mesure de ΔH.

La colonne 2 du tableau IV présente les moyennes des valeurs du rapport $N_4/N_{37}$ obtenues dans notre laboratoire sur 61 sérums normaux débarrassés de leur inhibiteur par chauffage à 56 C pendant 50 min (6) Dans la colonne 3 figurent les résultats obtenus

*Tableau IV*

| Génotype | Premières déterminations | EIGEL | SALMON |
|---|---|---|---|
| OO | 1,2 ± 0,07 | 1,19 ± 0,19 | |
| $A_1O$ | 2,02 | 2,08 ± 0,30 | |
| $A_2O$ | 2,54 ± 0,18 | 2,43 ± 0,28 | |
| $A_1A_1$ | 1,4 ± 0,11 | 1,44 ± 0,10 | |
| $A_2A_2$ | | 1,86 ± 0,07 | |
| Ax | | | 1,4 ± 0,1 |
| Am | | | 1,8 ± 0,1 |

récemment par EIGEL (9). Enfin on a ajouté les valeurs du rapport $N_A/N_{ST}$ d'après les recherches de SALMON (10, 11) sur des sous-groupes A de faible fréquence.

### *Homogénéité des isohémagglutinines normales*

Tous les résultats qui précèdent signifient évidemment que les sérums correspondant à un phénotype donné se comportent comme homogènes au point de vue de l'affinité des agglutinines. Comment autrement comprendrait-on les relations trouvées entre le génotype et des données purement expérimentales (tableaux I et II)?

Il peut être avantageux pour mettre bien en évidence l'homogénéité d'employer une relation linéaire pratiquement équivalente à (1) mais où les quantités d'agglutinine combinées sont rapportées non pas à une mole d'hématie mais à une mole d'agglutinine (12). En fait, plus de 75 % des agglutinines peuvent être combinées sans qu'apparaissent d'écarts à la linéarité. D'autre part, on a obtenu les mêmes pentes dans des expériences d'agglutination sur le sérum total, après une première absorption, et sur des élutions (12). Cette homogénéité des sérums normaux les distingue des immunsérums.

### *Les immunsérums*

Par absorption et élution des immunagglutinines on obtient des fractions qui, étudiées par la méthode thermodynamique, donnent des droites $N_t/N_f$ – $1/N'_4$ ayant des pentes différentes de celles obtenues avec les sérums non fractionnés (13, 14, 15). Le tableau V présente les valeurs obtenues dans des expériences effectuées avec des hématies $A_2$ mélangées soit à des sérums B0 et 0 normaux, soit à des sérums 0 stimulés par immunisation foeto-maternelle $A_1$.

*Tableau V (7, 8, 15)*

Les équilibres sont établis à 37° C en présence d'hématies $A_1$.
Les élutions sont pratiquées à 56° C après agglutination à 37° C.

| Agglutinines | | $(K_1/K_2)$ |
|---|---|---|
| α(B0) | Sérum total<br>Sérum absorbé<br>Elution | 12,4 ± 0,6 |
| α(0) | Sérum total<br>Sérum absorbé<br>Elution | 7,2 ± 0,3 |
| Immun-<br>-anti-A (0) | Sérum total<br>Sérum absorbé<br>Elution | de 1,3 à 3<br>de 3,8 à 8<br>de 0,2 à 1,5 |

Une étude thermodynamique analogue des anticorps anti-C et anti-D (Hughes – Jones *et al.* 16, 17) a mis en évidence leur hétérogénéité. Une distinction entre sérums normaux et immuns du système ABO avait été déjà observée par Boorman, Dodd et Morgan (18). On connaît maintenant d'autres caractères distinctifs qui se rencontrent éventuellement dans les sérums immuns. Les immunagglutinines réagissent avec la substance B de Gibbons et Morgan (19) dégradée par traitement à pH 8. Les hématies A de porc qui n'absorbent pas les isohémagglutinines normales fixent les agglutinines provoquées (15, 20). Enfin, Gibbs, Akeroyd *et coll.* (21) ont mis en évidence que les isoagglutinines normales sont inhibées par un extrait purifié de la muqueuse gastrique de porc, mais requièrent pour un même taux d'inhibition des doses de substances de porc beaucoup plus élevées que celles requises par les sérums anti A stimulés au moyen d'injections de substance A du porc.

*Poids moléculaires des agglutinines*

Nous avons pu montrer que les différences d'ordre énergétique liées au génotype sont en relation avec des différences de structure intéressant toute la molécule d'agglutinine. Des mesures d'ultracentrifugation analytique en cellule cloisonnée (22) ont révélé que les dimensions des molécules des agglutinines anti B, β(00), $\beta(A_1A_1)$, $\beta(A_10)$ diffèrent largement entre elles, les poids moléculaires moyens probables étant respectivement 170 000, 300 000 et 500 000.

Signalons à ce propos que RAWSON et ABELSON (23) par des fractionnements chromatographiques, ont vu que les agglutinines stimulées ou non stimulées des individus du groupe O ont des coefficients de sédimentation 7S alors que dans les sérums du groupe A il y a prédominance d isoagglutinines 19S D autre part, PROKOP (24) a interprété en se basant sur nos données les résultats de son étude sur la fréquence de la sécrétion d isoanticorps anti-B. Ayant trouvé cette fréquence supérieure chez les sujets du groupe O relativement à ceux des groupes $A_1$ et $A_2$, il attribue le fait à la plus grande diffusibilité des agglutinines d origine O.

Enfin BOWDLER et SWISHER (25) en séparant par chromatographie l agglutinine anti B d un sérum du groupe A la trouve confinée dans la portion contenant les $\gamma_1$-macroglobulines.

*Etudes des antigènes*

De même que la méthode thermodynamique nous renseigne sur les différences d affinité des agglutinines vis-à vis d un agglutinogène donné, elle sert à différencier les agglutinogènes quand on les fait réagir avec une agglutinine donnée. Les hématies peuvent se distinguer soit par la nature même des groupes auxquels s associe l'agglutinine, soit par le nombre $n$ de ces groupes accessibles sur une hématie.

Le tableau VI indique les données (7 8) permettant la comparaison des substances $A_1$ et $A_2$.

*Tableau VI* (7 8)
Combinaison avec l'isohémagglutinine (BO)

| | $A_1$ | $A_2$ |
|---|---|---|
| $-\Delta H$ (en Kcal) | 19 ± 3 | 20 ± 1,5 |
| Pentes à 25 C | 0,7 ± 0,07 | 3,3 ± 0,2 |

Dans ce cas, la différence entre $A_1$ et $A_2$ porte sur les pentes des droites ($N_i/N_f$ $1/N_A$) à même température – c est-à-dire sur les constantes d association avec l agglutinine $\alpha$(BO) – et non sur les variations de pentes avec la température c'est-à-dire sur les chaleurs de réaction.

En réunissant toutes les données de l étude thermodynamique on peut souvent interpréter certaines particularités de l agglutination. Ainsi, la chaleur de réaction de $A_2$ est plus grande avec une

agglutinine α d'origine B que d'origine O (tableau III). Quant à l'affinité, elle est plus forte à 37°C vis-à-vis de α(O) que vis-à-vis de α(B) (tableau II), tandis qu'à 25 C, cette proportion est inversée, comme conséquence de l'inégalité des chaleurs de réaction.

Notons ici, que l'on doit s'attendre à trouver de grandes irrégularités dans les expériences effectuées avec des hématies $A_1$ sans séparation préalable des isoagglutinines α et $α_1$ dont les affinités pour $A_1$ et les chaleurs de réaction sont très différentes (tableau III).

*En plus des données sur l'affinité des antigènes*, il est possible d'obtenir des mesures du nombre des groupes portés par une hématie. En effet, la connaissance, même approchée du poids moléculaire des agglutinines a permis au moyen de microdosages d'azote (26) de déterminer pour chaque espèce d'agglutinine la valeur du coefficient φ dans l'équation (1). On peut donc déterminer la valeur du nombre *n* en saturant d'agglutinine des hématies. On mesure la quantité d'agglutinine $N_A/φ$ à la fin de l'expérience. Le nombre des groupes agglutinogènes accessibles sur une hématie a été trouvé de l'ordre de 1 à 5 × 10³. Ce chiffre peut paraître élevé mais d'après des mesures effectuées sur la substance B de GIBBONS et MORGAN, il correspond à une composition raisonnable des hématies (13). D'ailleurs, dans une étude sur la combinaison d'une lectine avec les hématies humaines, BOYD *et al.* (27) ont été conduits à admettre que ce nombre peut être même dépassé quand des groupes agglutinogènes contenus à l'intérieur des hématies deviennent accessibles*. Nous trouverons dans les travaux de CH. SALMON que nous allons maintenant résumer d'intéressantes données sur les valeurs de *n* dans certaines variétés d'hématies.

### *Étude thermodynamique des sous-groupes sanguins de faible fréquence*

Une série de recherches concernant cette fois des groupes sanguins de faible fréquence a été entreprise par CH. SALMON. On doit à cet auteur d'importants résultats obtenus soit par la mesure de la quantité maximum d'agglutinine fixée par un nombre donné d'hématies, soit par application de la méthode thermodynamique.

Dans le premier cas (SALMON et REVIRON 28, 29), la quantité d'anticorps fixée dans les conditions de saturation était exprimée

* La détermination de *n* a servi à obtenir en valeur absolue les constantes d'association et de calculer les variations d'entropie (12); nous ne parlerons pas ici des *interprétations que nous* avons données de ces résultats.

en unités $N_4$, c est-à-dire mesurée par la différence ($N_4 - N_{\cdot 4} = N_f$) Trois phénotypes B faibles, $B_{80}$, $B_{60}$ et $B_{0}$ ont été définis, $B_{100}$ étant le phénotype normal. Les valeurs de $N_f$ sont respectivement 152,4 54 6, 3 1 277 6 unités $N_4$ En admettant que le nombre de molécules d agglutinine fixées par hématie est le même – soit 1600 environ* – que dans les expériences de détermination de $N_4$ de FILITTI WURMSER *et al.* (3) pour l agglutinine $\beta(A_1O)$ à 4 C, le nombre des sites d antigène présents sur une cellule peut être évalué. On trouve pour $B_{80}$, $B_{60}$, $B_{0}$ respectivement 200 000 70 000 et 4000. Les auteurs font l'hypothèse que la quantité d antigène synthétisée dans l érythroblaste et l érythrocyte pourrait être directement lié à la quantité de matériel actif, et la relation entre les chiffres observés 277 152 55 et 3 pourrait alors indiquer que ces variations représentent des multiples d unités actives.

Dans son application de la méthode thermodynamique, SALMON a utilisé une transformation de la relation (1) qu il a mise sous la forme

$$\frac{N_4}{N_{\cdot 4}} = 1 + \left(\frac{1}{\alpha} \quad N_t \quad 10^{-8}\right)$$

telle que l on ait, en ordonnée, une valeur aléatoire, $N_4/N_{\cdot 4}$ et en abscisse une valeur contrôlée, $N_t$ cette transformation permet le contrôle statistique par la méthode des moindres carrés. La pente $1/\alpha$ déterminée de cette manière, est l inverse de la pente $1/nK$ de l équation (1) mais ceci ne changeant rien à l'interprétation des résultats. On obtient alors une droite passant par le point 1 de l ordonnée, dont la pente est dépendante de l affinité. Les résultats sont indiqués dans le tableau VII (29)

SALMON fait remarquer qu'il est difficile d interpréter les variations observées dans la pente pour d un même anticorps, étant donné que la pente est elle-même liée au nombre des sites antigènes. Par contre, les différences de chaleur de réaction observées pour un même anticorps, entre les hématies «$B_{100}$» et les deux types d'hématies $B_{80}$ et $B_{0}$ étudiés, impliquent qu en plus des différences quantitatives, il existe, entre ces trois variétés d antigènes B de profondes différences de structure probablement liées à des variations dans la configuration des groupes spécifiques.

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* Pour $\beta(A_1O)$ à 4 C l'agglutinine restée libre quand le maximum d'hématies agglutinées est atteint est d'environ 1 p. 100 de l'agglutinine totale. Si le taux d'agglutination est 0,45, on [illegible] pratiquement pour le nombre de molécules fixées sur une hématie ($6 \times 10^{?} \times 0{,}45$) / $\varphi A_1O$, soit environ 1 600.

*Tableau VII*

| | Agglutinabilité (1) | Quantité d'antigènes (2) | Affinité d'un anti-B de sujet 0 (COU) (3) 37° C | 25° C | 4 C | −ΔH cal/mol (4) |
|---|---|---|---|---|---|---|
| B normal | 95-99 | 277,6 | 2,17 | 2,39 | 3,38 | 2 900 |
| $A_1$ B APC | 80 | 152,5 | nt | nt | nt | nt |
| B PAN | 53,8 | 47,6 | nt | 1,02 | 7,30 | 15 300 |
| B CAL | 57,8 | 52,9 | | | | |
| B BIL | 64 4 | 63,3 | nt | nt | nt | |
| B MAL | 0 | 3,1 | nt | 0,20 | 1,03 | 12 700 |

(1) % de cellules agglutinées par $10^6$ unités N d'anticorps anti-B dans une suspension de 500 000 hématies par mm³

(2) Nombre maximum d'unités N d'anticorps anti-B que l'on peut fixer à la surface d'une hématie

(3) Valeur de la pente $P = \frac{(N_1/N_4) - 1}{N_t \quad 10}$

(4) Calculée à partir des pentes d'après l'équation de Van t'Hoff

n(t) : non testé.

## *Méthodes des probits*

*Un important groupe de travaux a été accompli aux Etats* Unis, en employant une technique de dosage des agglutinines due à Wilkie et Becker (30 31) Cette technique, comme la mesure du $N_4$ repose sur un comptage des cellules non agglutinées. On mesure le taux d agglutination dans des mélanges d une quantité constante de cellules avec des sérums de dilution croissante. Si l on porte le taux en fonction de la concentration on obtient une courbe d allure sigmoïde. Wilkie et Becker, pour avoir une relation linéaire, substituent au taux d agglutination les probits correspondants. La valeur $HD_{50}$ pour laquelle le probit est égal à 5 mesure la concentration de l antisérum qui agglutine 50 % d une suspension de cellules de référence. La méthode est actuellement très largement appliquée, avec souvent utilisation d un compteur électronique de particules (32–38) Ce dernier perfectionnement amène d ailleurs à opérer avec des sérums extrêmement dilués d où peuvent résulter des causes d erreur – dénaturation, transconformation moléculaire – qui ne sont nullement inhérentes à la méthode.

Jusqu à présent, la technique de Wilkie et Becker a été surtout utilisée en interprétant, soit la mesure de $HD_{50}$, soit la pente de la ligne de régression probit-log concentration du sérum, c est-à dire l accroissement d agglutination résultant de l accroissement

d une unité de concentration. GIBBS et AKEROYD (40) qui ont été les premiers à utiliser systématiquement la méthode de WILKIE et BECKER ont été également les premiers à souligner les difficultés d interprétation en l absence d une théorie bien fondée de la ligne de régression.

Effectivement, le phénomène total de l agglutination est très complexe, bien que dans un certain domaine de composition des mélanges de sérum et d'hématies, quand le nombre $N_t$ de ces der mères reste constant, on observe que le taux d agglutination aug mente proportionnellement au nombre moyen de molécules d ag glutinines fixées par hématie. On peut montrer alors que les dilu tions que l'on fait subir aux sérums pour avoir des taux d agglutina tion mesurables compensent plus ou moins complètement les diffé rences d affinité et de nombre des groupes. Bien entendu, à ces re-lations se superposent des complications qui naissent des processus secondaires d agrégation. Il en résulte que la signification des lignes de régression probit-log concentration des sérums n est pas encore comprise complètement. Mais nous pensons que les différen-ces de pente, en l absence d une autre donnée complémentaire, ne peuvent pas être attribuées avec certitude à des différences quali tatives.

En ce qui concerne les processus secondaires de l agglutination qui suivent la combinaison avec l'agglutinine, signalons l'analyse qui en a été faite par BOWDLER et SWISHER (25) Un point impor tant a trait à la réversibilité de l'aggrégation. WILKIE et BECKER (31) avaient confirmé par des expériences d agrégation et désagré-gation le réversibilité de l agglutination pour les sérums anti B trouvée par FILITTI WURMSER et JACQUOT ARMAND Or GIBBS et AKEROYD (39) constatèrent dans des expériences analogues qu un état final exactement le même n était pas obtenu suivant que se formait ou se défaisait un agrégat lorsque l on met en présence des hématies A avec des sérums O WILKIE (41) trouva un fait sembla ble en étudiant l action anti B de ces sérums. Cet effet, dû à quel que irréversibilité dans la formation des grands agrégats, n inter vient pas dans l emploi de la méthode thermodynamique basée sur la réversibilité de la première phase de l agglutination, l'énergie correspondant à la partie irréversible de la deuxième phase étant certainement négligeable. Une certaine irréversibilité peut aussi provenir de la présence d'immunanticorps à double spécificité de haute affinité (SOLOMON [42])

Malgré les difficultés d'interprétation des différences observées dans les pentes de la droite probit log concentration d'intéressants résultats en ont été tirés. Dans l'ensemble, chaque fois que la méthode le permet, il y a concordance avec ce que nous a appris la méthode thermodynamique par mesure des $N_1$ bien que les conditions de concentration dans lesquelles les deux méthodes ont été employées soient très différentes et que la phase d'agglutination apporte une source d'irrégularités.

Dans leurs travaux sur l'immunagglutinine anti-A, GIBBS et AKEROYD (39 40) ont obtenu, avec les hématies $A_1$, $A_1B$, $A_2$, $A_{int}B$, $A_2B$, des différences de pentes des droites probit log concentration qu'ils attribuent à des différences qualitatives. Nous avons vu plus haut l'incertitude de la signification de ces pentes. Elle ne nous paraît pouvoir être levée que par des mesures du nombre des groupes et de leur affinité. Cependant dans le cas particulier des antigènes $A_1$ et $A_2$ rappelons que MAVRIDES (7) a effectivement montré que les constantes d'association des isoagglutinines α sont différentes (tableau II)

GIBBS *et al.* (44) ont aussi trouvé des différences qu'ils pensent cette fois quantitatives — à cause du parallélisme des lignes de régression — plutôt que qualitatives entre des sous-groupes d'antigènes B dans trois groupes raciaux. Citons encore les observations de GIBBS *et al.* (43)

Enfin, des différences de pente des lignes de régression ont été montrées par WILKIE et BECKER (31) dans une comparaison entre les hématies BB et $A_1B$.

La méthode des probits a encore servi à l'étude de plusieurs phénotypes $A^h_m$ et $0^h_m$ (SOLOMON *et al.* [45]) $A_{el}$ (SOLOMON et STURGEON [46]) Enfin, appliquée à la réaction des extraits anti H de ULEX avec les hématies humaines, elle a révélé des différences entre les hématies de divers groupes (SOLOMON [47])

En résumé, des résultats fort intéressants peuvent être obtenus par la simple mesure du taux d'agglutination, mais l'interprétation en est toujours délicate, pour les raisons données plus haut concernant la distinction entre différence de quantité et de nature, distinction que la méthode thermodynamique apporte de manière non équivoque. En plus des causes d'erreur communes aux deux méthodes, (présence d'immunagglutinines ou d'inhibiteurs) il existe celles (dénaturation, transconformations) qui proviennent de l'extrême dilution à laquelle sont souvent effectuées les mesures.

*Biosynthèse des isoagglutinines*

Le fait essentiel qui a permis de dégager l'étude thermodynamique des isoagglutinines nous paraît être l'existence d'une spécificité de certaines protéines sériques à l'intérieur de l'espèce humaine. Les isoagglutinines $\beta$ en ont été les premiers exemples et l'isoagglutinine $\beta(A_1O)$ la première protéine hybride humaine connue (48).

On a à considérer dans ces anticorps une double spécificité : spécificité vis-à-vis de l'antigène et structure protéique caractéristique du génotype d'origine. La synthèse est-elle alors entièrement dépendante de la commande directe des gènes ou, comme l'a suggéré J. B. S. Haldane (49) dépend-elle en partie de la présence de l'antigène? Un argument en faveur de cette dernière hypothèse est la mise en évidence (50) de la structure $\beta(A_1O)$ pour l'isoagglutinine anti B dans le sérum d'une femme, une «chimère» dont les hématies sont de deux sortes : 61 % du groupe O et 39 % du groupe $A_1$. Un autre fait découvert par Salmon (51) est la constatation que des modifications d'antigène A, qui surviennent sur les hématies de certaines leucémies aiguës, s'accompagnent d'anomalies parallèles *des propriétés thermodynamiques de l'anti B.* Chez un sujet $A_1O$ leucémique dont les hématies n'ont pas subi de modification ABH, l'affinité de l'anticorps anti B est celle d'un $A_1O$ normal. Au contraire, chez un sujet $A_1O$ leucémique dont l'antigène A a subi une modification, l'affinité de l'anticorps anti B est très faible, et tout à fait anormale pour un phénotype $A_1O$. Salmon (52) a pu faire une expérience qui paraît établir solidement le rôle de l'antigène circulant. Chez un enfant érythroblastopénique de groupe $A_1$ il a pu alterner les transfusions de globules rouges lavés de groupe $A_1O$ et de groupe O. Il a alors mesuré l'affinité de l'anticorps anti B formé après chaque série de transfusion. Les valeurs des pentes $([N/N_{s}]-1)/N_{i} \times 10^{-8}$ observées, après transfusion de globules rouges $A_1O$ correspondent bien aux valeurs attendues d'un anti B type $\beta(A_1O)$. Au contraire, les valeurs observées après transfusion de globules rouges O lavés sont très différentes des précédentes. Ces résultats prouvent que l'affinité de l'anticorps anti-B, liée à la structure de la molécule de globuline spécifique, est différente selon la nature de l'antigène ABH des hématies en circulation.

Diverses hypothèses peuvent être avancées sur la manière dont la synthèse des isoagglutinines reçoivent l'information correspondant à leur double spécificité. La présence d'un antigène donné

peut influer sur un certain gène contrôlant un système de régulation comme il a été supposé pour la synthèse d'isocytochromes (SLONIMSKI *et al.* [53]). On peut aussi supposer que l'antigène intervient à un stade cytoplasmique.

Cette technologie de la formation d'une protéine spécifique ne peut encore donner lieu qu'à des échanges d'idées. Il nous paraît cependant devoir être noté qu'à ce point de vue, les modifications obtenues après les transfusions de cellules $A_1O$ et O effectuées par SALMON constituent un type d'expérimentation nouveau et prometteur.

## *Résumé*

L'étude thermodynamique de la combinaison des agglutinines avec les agglutinogènes permet de spécifier si les différences observées dans l'agglutination par divers sérums de cellules portant le même antigène sont quantitatives ou qualitatives. Il a été montré que l'affinité d'une isohémagglutinine humaine permanente du système A B H dépend du génotype de l'individu qui l'  formée. On expose les résultats de l'application de la méthode à l'étude des immunagglutinines, des antigènes et, en particulier des sous-groupes sanguins de faible fréquence, ainsi que des modifications pathologiques. – Les travaux basés sur l'interprétation des diagrammes de probit sont passés en revue. – Le mécanisme de la biosynthèse des isohémagglutinines est discuté.

## *Summary*

A thermodynamic approach to the study of the combination of agglutinins with agglutinogens permits to specify wether observed differences in the agglutination by various sera of cells containing the same antigen are quantitative or qualitative. It is shown that the affinity of human permanent isohaemagglutinin of the ABH system depends on the genotype of the person who has produced it.—The results of the application of the method to the study of immunagglutinins, antigens, and particularly of infrequent blood subgroups and pathologic modifications summarized.—Work based on the interpretation of probit plots is reviewed.—Mechanism of isohaemagglutinin biosynthesis is discussed.

## *Zusammenfassung*

Die thermodynamische Untersuchung gestattet zu unterscheiden, ob die beobachteten Unterschiede in der Agglutination durch verschiedene Sera von Zellen, welche Träger desselben Antigen sind, quantitativer oder qualitativer Art sind. Man zeigt, daß die Affinität eines menschlichen permanenten (natürlichen) Isohämagglutinins im ABO-System vom Genotyp des Individuums abhängt, der es produziert hat. – Die Resultate der Anwendung dieser Methode in Untersuchungen von Immunagglutininen, Antigenen und besonders von selteneren Blutuntergruppen und von pathologischen Veränderungen werden gegeben. – Man gibt auch eine Übersicht der Interpretation der Probit-Diagramme und diskutiert den Mechanismus der Biosynthese der Isohämagglutinine.

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# Thalassaemia

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Thalassaemia is derived from the word θάλασσα the Sea, or in the classical meaning the Mediterranean. The word is an etymological monstrosity and indicates to the Greek 'Sea in the Blood" rather than Mediterranean anaemia, which it was supposed to denote. Also, it is altogether a misnomer because, although the first observations on this anaemia were made in Mediterranean populations, it is now known that this condition is spread all over the world and that there must be more "thalassaemics" in China with its vast population than there are Mediterraneans altogether

## *Thalassaemia Major and Minor*

In 1925 COOLEY and LEE described the severe form now known as *thalassaemia major this work was carried out in North America.* At about the same time work in Italy and Greece prepared the ground for the recognition that there were two similar familiar conditions which varied in severity The major disability was found in the homozygous state. The milder condition was also described in the United States where it was named target cell anaemia or familiar microcytic anaemia. Here the lesser severity was found when the gene responsible for the abnormality was inherited as a single dominant trait. A paper which conclusively proved the mode of inheritance was published by VALENTINE and NEEL in 1944 and became so important because it was later used as the model for the investigation of the sickle-cell anaemia.

The terms thalassaemia major and thalassaemia minor have been used in two different ways. To the geneticist the first describes the homozygous and the second the heterozygous state and this is today the current usage. However clinicians have liked to use the words major and minor to describe clinical severity and indeed the term thalassaemia intermedia was introduced to describe those disease states, which were midway in clinical morbidity between

thalassaemia major and minor The term thalassaemia minima even has been used, when the clinical condition was so near normal that it did not seem to deserve the description of thalassaemia minor

The clinical picture in thalassaemia major is uniformly severe and results invariably in death in childhood. In contrast there is a great deal of variety in the clinical severity of thalassaemia minor Patients who are well nourished and who do not suffer from infections and lead a life free from stress, may be quite capable of compensating the shortened red cell life span of thalassaemia minor

A combination of the heterozygous state for Haemoglobin S and $\beta$ thalassaemia causes an anaemia but this is usually less severe than the clinical condition found in homozygous sickle-cell disease.

*The Haematological Abnormality*

The classical Mediterranean anaemia is caused by a shortage of adult haemoglobin and it is thought that this is the outcome of a disturbance of the function of messenger RNA in carrying the genetic information from nuclear DNA to the cytoplasmic ribosomes manufacturing adult globin. Thus, in the earlier months of life, when most of the haemoglobin is still Haemoglobin F the child is protected.

When Haemoglobin C was discovered the blood of several American Negro patients, who had been diagnosed as suffering from thalassaemia, was re-examined. A number of them were found to be suffering from Haemoglobin C disease and not from thalassaemia. It was suggested that thalassaemia may be a haemoglobinopathy resulting from an abnormal haemoglobin which could not be recognised. It is just possible that if the great pool of thalassaemics known to occur all over the world were examined by a new technique one might find amongst them other types of haemoglobinopathy—especially perhaps amino-acid substitutions, which do not give the altered haemoglobin molecule a change in charge and therefore are not detectable by electrophoresis.

However for the great majority of thalassaemia cases it is no longer thought that an abnormal haemoglobin is responsible. KOTUOBARRO and GUIDOTTI have isolated the Haemoglobin A from patients with sickle-cell thalassaemia and Haemoglobin C thalassaemia and examined the amino-acid sequence they found it was identical with that of Haemoglobin A.

The basic disability in thalassaemia is therefore not the production of an abnormal haemoglobin but a failure to produce red cells with a normal adult haemoglobin content. The appearance of the red cells in Mediterranean anaemia is perhaps the most constant diagnostic feature because they vary in size and some are very thin and pale. Thus thalassaemia, like iron deficiency is also a hypochromic anaemia. Hypochromia denotes a pallor of the red cells and indicates a deficiency of haemoglobin such a deficiency can arise either from lack of iron, as for example in iron deficiency anaemia, in which case the bottleneck would be the haem production, or from a deficiency of globin, as in thalassaemia, in which case the bottleneck is the manufacture of the protein *moiety* In thalassaemia, any abnormalities of haem metabolism which occur are secondary and arise merely because haem accumulates and cannot be utilised.

There is one significant difference between the hypochromia of severe iron deficiency anaemia and of thalassaemia. In both conditions the cells are hypochromic but the mean corpuscular haemoglobin concentration (M.C.H.C.) in iron deficiency anaemia is very much lower than in thalassaemia. It is possible that in iron deficiency a certain amount of colourless globin, to which haem is not attached, increases the ratio of non-haemoglobin to haemoglobin within the red cell, so that the cell contents are not as reduced as in the red cells of thalassaemia. In thalassaemia, where there is limitation of globulin manufacture, such a functionless and colourless packing could not be possible Thus it follows that the mean corpuscular haemoglobin is low both in thalassaemia and in iron deficiency but that the mean haemoglobin concentration is lower in iron deficiency anaemia.

These thin, abnormally shaped cells have a shortened life span and the resulting chronic haemolysis results in a compensatory over-activity of the bone marrow This is reflected in the presence of nucleated red cells in the peripheral blood and in an increase in reticulocytes. Prankerd has shown that effective red cell production per unit of active bone marrow is decreased in thalassaemia but the total number of red cells entering the blood is greater than normal because the volume of active bone marrow has been considerably expanded. Destruction of red cells is accelerated, and whether the anaemia will be more or less severe will depend upon the equilibrium the patient has established between increased pro-

duction and increased destruction. However at whatever level this equilibrium is finally placed it cannot be stable and any depression of the bone marrow activity would tend to disturb the delicately balanced state. This often happens in debilitating diseases and the same will occur when the body is faced with additional demands as in pregnancy.

The fundamental feature of thalassaemia is this inability to produce enough Haemoglobin A. This lack of Haemoglobin A results in abnormally shaped cells, and these do not seem to be well suited to survive the buffetting that a normal red cell receives. A red cell can expect to travel about 700 miles during its lifetime and unusually thin cells fall by the wayside during this turbulant journey. This failure to produce Haemoglobin A ($\alpha_2\beta_2$) may be due to an inability to produce either the $\alpha$ or the $\beta$ type polypeptied chains. It follows, therefore, that there are two types of thalassaemia, $\alpha$ thalassaemia and $\beta$ thalassaemia, the latter being the classical Mediterranean anaemia. $\alpha$ thalassaemia will depress only the production of the $\alpha$ chains and $\beta$ thalassaemia will depress only the production of the $\beta$ chains. Both $\alpha$ and $\beta$ thalassaemia may occur in a major (homozygous) and minor (heterozygous) form.

There is another type of $\alpha$ thalassaemia which is called Haemoglobin H disease. In this condition we have a combination of heterozygous $\alpha$ thalassaemia and a third gene which on its own is qui undetectable. Such a combination results in an anaemia which ma on occasions be sufficiently severe to cause death. It is appa therefore that the combination of one gene for $\alpha$ thalassaemia w an otherwise completely silent gene can result in a pronou deficiency of $\alpha$ chain production.

Of quite a different nature is of course, the interac thalassaemia and abnormal haemoglobins.

## Clinical Course

$\beta$ Thalassaemia major $\beta$ thalassaemia minor $\alpha$ tha major and $\alpha$ thalassaemia minor are conditions whic clinical states of widely different severity $\alpha$ Thalassa incompatible with life and results in still-birth. $\beta$ Thala gives a very severe anaemia with death in childhood. $\beta$ T minor although commonly associated with a ha

between 10 and 12 g per 100 ml, varies in severity between disability due to anaemia and an asymptomatic condition. *α* thalassaemia minor does not give rise to anaemia and indeed is very difficult to recognise. Haemoglobin H disease gives a clinical picture which lies usually midway between *β* thalassaemia minor and major. In discussing the clinical presentation of thalassaemia it is therefore necessary to consider these various sub-divisions separately.

### *β Thalassaemia Major*

*Anyone afflicted with β thalassaemia major can be expected* to die in childhood. The impairment of function of both the *β* chain loci proves too great a handicap for survival, their haemoglobin level very seldom being above 7 g %.

As the basic difficulty is in the production of *β* chains, only Haemoglobin A will be depressed because this haemoglobin ($\alpha_2\beta_2$) is the only pigment which contains *β* chains.

The first symptoms are therefore seen when Haemoglobin A production becomes predominant at the age of 3 to 5 months. Despite the fact that the anaemia is insidious in onset, the parents rarely give such a history. The usual story is that on one day there was a thunderstorm or the baby was dropped or it refused to eat or had diarrhoea and that from this day onwards the baby looked pale and never was the same again. Anaemia soon becomes pronounced and splenomegaly and, with a lesser regularity hepatomegaly may be noticed early. The spleen may enlarge sufficiently to cause a noticeable abdominal swelling. The few children who survive to the age of 12 or 14 are physically and sexually undeveloped and occasionally have leg ulcers.

The marrow hypertrophy causes characteristic X-ray changes in the skeleton. In the skull, for instance, there is wide separation and thinning of both the inner and outer *tables* brought about by *expansion of the diploe. To prevent the bony structure from col*lapsing the marrow is permeated with thin spicules of bone suggestive of the strengthening of reinforced concrete by wires. In the skull the perpendicular spicules give the characteristic X ray picture of 'hair on end'. The thickening of the cranial bones, together with the relatively sunken nose, result in a Mongoloid facies.

The X-ray changes are not a specific feature of thalassaemia and merely represent the reaction of the marrow to the necessity

for increased red cell formation. Thus similar X ray changes may be noted in hereditary spherocytic anaemia and the anaemia of congenital syphilis, both of which conditions also have their onset in early childhood.

Normal erythropoiesis proceeds at a pace which does not make very great demands upon the marrow space. The adult has active "red" marrow only in the skull, spine, thoracic cage and pelvis. With the exceptions of the top of the femur and humerus, the marrow in the bones of the legs and arms is filled with fat and looks yellow Thus only about half the marrow volume available is utilised for erythropoies. Even this half, in fact, is not fully used because up to half of the red marrow of a normal person may be fat. A new born in contrast has red marrow also in the limbs and it is possible therefore to obtain a satisfactory diagnostic sample of active marrow from a puncture of the tibia. The marrow regression begins at about 5 years and the adult pattern is fully established by late adolescence. It follows that if there is a comparatively mild haemolytic process it is possible for the adult to call upon his reserve spaces and convert them into active sites for red cell production. Such a person would then be able to sustain a satisfactory level of haemoglobin although he may in fact be suffering from an increased haemolysis. This is the so-called compensated haemolytic anaemia, which strictly speaking is not an anaemia at all. When, as in thalassaemia major the haemolytic process is too severe, even the utilisation of the total marrow space will be inadequate and frank anaemia will result. It must be remembered that the young child already has all the mar row space occupied by red marrow After the few fat spaces in the red marrow have been used, expansion can only occur by increasing the volume of the marrow cavity by erosion of the surrounding bones.

*β Thalassaemia Minor*

β Thalassaemia minor appears to be the most frequent form of thalassaemia and is spread all over the globe. In some areas of Italy more than 20% of the population have β thalassaemia minor We know that, when two heterozygotes for β thalassaemia marry one quarter of the offspring will be homozygotes for the thalassaemia gene and therefore have thalassaemia major and die before the reproductive age. It will suffice to say at this stage that if the heterozygous state did not have some advantages this particular

mutation would be rapidly eliminated by natural selection. It has already been mentioned that the patient with $\beta$ thalassaemia minor is on the whole nearly as fit as his unafflicted neighbour There may be a mild anaemia (Hb 10–12 g per 100 ml) which causes little or no disability and the spleen may be palpable. The X ray abnormalities described in the previous section may be present in some cases to a minor degree. As with any chronic haemolytic process there is an increased liability to form bilirubin stones in the gall bladder

These apparently fit people may occasionally present with severe anaemia when additional strain is thrown on to their erythropoietic system. $\beta$ Thalassaemia minor becomes quite severe haemolytic anaemia when associated with malnutrition, parasitic infestations or chronic infections. There may be periods when in an individual the disease may change in severity a healthy strapping young girl may suddenly develop quite a significant anaemia when she becomes pregnant. The clinical state of a patient with $\beta$ thalassaemia minor may thus vary between normality and severe haemolytic anaemia. They live as it were on the edge of a volcano and it is the duty of the physician to listen out for the early rumblings that may spell subsequent disaster

### *α Thalassaemia Major*

The homozygous state for the $\alpha$ thalassaemia gene is incompatible with life. When both lines of $\alpha$ chain production are affected it is obvious that the compensation of one for the other cannot come into play The condition is lethal and produces hydrops foetalis with the miscarriage of a non viable child during pregnancy

### *α Thalassaemia Minor*

Because the heterozygous state for $\alpha$ thalassaemia is absolutely harmless, one must presume that one gene for $\alpha$ chain production is capable of doing practically the work for both.

### *Haemoglobin H Disease*

The severity of Haemoglobin H disease lies between $\beta$ thalassaemia minor and major and it varies between a barely compensated and a frank haemolytic anaemia. The patients may well have a

permanent slight anaemia in the region of 10 g per 100 ml which is accentuated in times of adversity just as in the case with $\beta$ thalassaemia minor A woman with Haemoglobin H disease is usually able to bear children, but like all patients with this condition reacts badly to infection and easily becomes folic acid deficient because of the increased red cell production.

## Diagnosis of Thalassaemia

### *$\beta$ Thalassaemia Major*

The haemoglobin level of afflicted children lies between 3 and 7 g per 100 ml and, as with any severe haemolytic process, the child shows nucleated red cells and reticulocytes in the peripheral blood. The intense activity of the bone marrow although mainly erythropoietic, is also leucopoietic, giving in some cases a leukaemoid peripheral blood picture. Although the platelet count is normal, it is not unusual for a child to be provisionally diagnosed as a case of leukaemia. This is particularly liable to occur because one is likely to find a raised level of foetal haemoglobin both in young children with thalassaemia and young children with leukaemia. Abnormally shaped red cells (poikilocytes) and basophilic stippling are also frequently found. Because the cells are hypochromic they can be recognised *in vitro* by their increased resistance to lysis by hypotonic saline solution. Some of the very thin cells give the appearance of a target or a Mexican hat because there is so little haemoglobin that the red cell membrane touches, with the exception of a central area and also around the rim. It has already been mentioned that the mean corpuscular haemoglobin concentration (M.C.H.C.) in the red cells will be usually somewhat below normal and the mean corpuscular haemoglobin (M.C.H.) will be greatly reduced. The association of a low mean corpuscular volume (M.C.V.) with only a very moderate reduction of the mean corpuscular haemoglobin concentration (M.C.H.C.) indicates thalassaemia rather than iron deficiency Indeed in some cases the M.C.H.C. may be normal or near normal. Unfortunately the inaccuracy of the red cell count, without electronic equipment, greatly reduces the value of the M.C.H. and M.C.V in the diagnosis of haematological disorders. In thalassaemia the serum iron is increased, provided no secondary iron deficiency is present, and indeed the iron binding protein may be totally saturated. The marrow itself shows excessive iron deposits.

As the basic cause of this condition is $\beta$ chain deficiency Haemoglobin $A_2$ ($\alpha_2\delta_2$) and Haemoglobin F ($\alpha_2\gamma_2$) are not directly affected and it is perhaps not surprising that these two haemoglobins show a relative rise in thalassaemia. Examination of the haemoglobin would show that foetal haemoglobin in thalassaemia major may consist of 15% to 90% of the total and adult haemoglobin from 20% to 80% of the total. Although the proportion of Haemoglobin $A_2$ may not be raised if compared with total haemoblobin (A + F) present, it will be in fact found to be high if it is related to the Haemoglobin A alone.

It is an obvious requisite for the diagnosis of $\beta$ thalassaemia major that both parents should have $\beta$ thalassaemia minor. The possibility of illegitimacy should not be overlooked and in apparent discrepant cases blood grouping may be of value. Although the gene is extremely widespread geographically the parents would in the Old World commonly originate from the belt which stretches from the Mediterrean across the middle and far east to China.

In practice the clinician seldom finds difficulty in diagnosing $\beta$ thalassaemia major. In any case the parents are often only too well aware of the nature of their child's disease.

### *$\beta$ Thalassaemia Minor*

The practical problem in the diagnosis of thalassaemia is the diagnosis of $\beta$ thalassaemia minor. The first requirements for the diagnosis is the exclusion of iron deficiency because, when iron deficiency is found, a co-existent thalassaemia cannot be completely excluded. This is particularly important both in the diagnosis of Haemoglobin H disease, where it will be separately considered, and in mass surveys where osmotic fragility is used as a screening procedure and iron deficiency gives false positive results for thalassaemia. We have already mentioned that the degree of anaemia found in $\beta$ thalassaemia minor is very variable. The haematological findings therefore tend to lie anywhere between the gross changes of thalassaemia major and normality. A young man living on a good mixed diet may well have a haemoglobin level within the normal range. In contrast, a woman living under poor conditions may have a degree of anaemia one would normally associate with $\beta$ thalassaemia major. In any case an examination of the blood smear tends to show greater changes than one would expect from the

haemoglobin level. Target cells may be present, together with basophilic stippling and some poikilocytosis and polychromasia. Rather more than half the cases of $\beta$ thalassaemia minor have a raised foetal haemoglobin lying usually in the range of 2% to 5% which can easily be demonstrated by measuring the alkali resistance of haemoglobin. The raised Haemoglobin $A_2$ which is present in the great majority of cases can be demonstrated by paper electrophoresis using a Tris buffer or by starch gel electrophoresis. If Haemoglobin $A_2$ is not raised, one now assumes that the primary deficiency is not in the $\beta$ chain production but in the $\delta$ chain production, and that the $\beta$ chain is only secondarily affected. The search for Haemoglobin $A_2$ by electrophoresis would at the same time demonstrate abnormal haemoglobins which might be present. If such an abnormal haemoglobin is present its proportion can also be ascertained. This is of value when one is dealing with a case of interaction between thalassaemia and an abnormal haemoglobin. To place the diagnosis beyond any doubt it is essential to perform a family study. An estimation of serum iron is valuable but it is important to keep in mind that a low serum iron does not necessarily exclude thalassaemia. This is particularly relevant when one is diagnosing $\beta$ thalassaemia minor in a woman who has had many children and is therefore particularly liable to have iron deficiency.

In the screening of a population, simple tests for resistance of the red cells to hypotonic saline solution is often used. This in fact merely detects hypochromia and will discover equally thalassaemia and iron deficiency. It is obvious that this screening test has to be followed by further investigations before the diagnosis of thalassaemia can be assumed. Despite this limitation the demonstration of increased resistance of the red cells to hypotonic saline is a valuable diagnostic test for thalassaemia.

### $\alpha$ *Thalassaemia Major*

Thalassaemia is a disease which results not from a total gene deletion but from a partial gene supression. In $\alpha$ thalassaemia major both $\alpha$ chain genes are affected and neither is able to produce an adequate quantity of $\alpha$ chains. Though the haemoglobin of a still-born foetus consists mostly of Haemoglobin Barts ($\gamma_4$) some $\alpha$ chains are formed and a very small proportion of Haemoglobin F ($\alpha_2\gamma_2$) and even lesser part of Haemoglobin A ($\alpha_2\beta_2$) may be found.

Some traces of Haemoglobin H ($\beta_4$) are always present because $\beta$ chain production has already commenced

Examination of parents might demonstrate $\alpha$ thalassaemia minor but the difficulties of reaching this diagnosis are discussed in the following section.

*$\alpha$ Thalassaemia Minor*

The heterozygous state for $\alpha$ thalassaemia minor is recognised *during survey work by the discovery of Haemoglobin Barts* ($\gamma_4$) *in* a newly born child by itself not necessarily specific, and by the observation that the red cells of these children, when they grow up, have a tendency to microcytosis and some increased resistance to hypotonic saline solution. The parents also may show minimal *features of a thalassaemic blood picture. However there are no* abnormalities of haemoglobin production in the adult, and in the child no Haemoglobin H ($\beta_4$) will be found when Haemoglobin Barts disappears a few months after birth.

Diagnosis of $\alpha$ thalassaemia trait is therefore difficult Not only is the heterozygous state for $\alpha$ thalassaemia absolutely symptomless but also it is very difficult to recognise haematologically As a result these carriers of the $\alpha$ trait are clinically indistinguishable from normal.

*Haemoglobin H Disease*

Much of what was stated about the diagnosis of $\beta$ thalassaemia minor is also true of Haemoglobin H disease, particularly the variable level of the haemoglobin and the findings on the blood film. However as Haemoglobin H disease is a deficiency of $\alpha$ chain production, this will affect the production of all three haemoglobins, A ($\alpha_2\beta_2$) F ($\alpha_2\gamma_2$) and $A_2$ ($\alpha_2\delta_2$) One cannot expect a high Haemoglobin in F or Haemoglobin $A_2$ in an $\alpha$ chain deficiency disease. On the contrary in the competition for $\alpha$ chains the $\beta$ chains are more avid than the $\gamma$ chains and $\delta$ chains. It follows therefore that when there is a shortage of $\alpha$ chains Haemoglobin F would disappear earlier in life than usual, and Haemoglobin $A_2$ will be decreased rather than increased or normal. The depression in the $\alpha$ chain production manifests itself by the formation of the tetramers Haemoglobin Barts ($\gamma_4$) in the infant, and Haemoglobin H ($\beta_4$) in the older child and adult. Neither of these two haemoglobins require

any $\alpha$ chains. Because Haemoglobin H disease in clinical severity is somewhat similar to that of $\beta$ thalassaemia minor it also will often remain undiscovered.

The correction of iron deficiency prior to diagnostic testing is particularly important in the case of Haemoglobin H disease. If the bottleneck in the production of haemoglobin is the manufacture of the haem, as in iron deficiency then even in Haemoglobin H disease sufficient $\alpha$ chains can be produced to saturate the reduced amounts of haem which are made available. No Haemoglobin H would then be formed because under the conditions of iron deficiency there is no relative shortage of $\alpha$ chains—there is only a shortage of haem groups. When, however the iron deficiency is corrected, the $\alpha$ chain deficiency becomes unmasked because haem production is no longer the bottleneck, and the limiting factor becomes the rate at which $\alpha$ chains are made available. Under these conditions some of the free haem groups are mopped up by the readily available $\beta$ chains which form the tetramer H ($\beta_4$) Thus, when Haemoglobin H disease is suspected and iron deficiency anaemia is diagnosed, the haemoglobin should be re-investigated after the iron deficiency has been corrected.

When iron deficiency and Haemoglobin H disease co-exist, the abnormal diagnostic fraction for Haemoglobin H ($\beta_4$) may disappear The laboratory is not faced with quite the same difficulty in the case of the combination of iron deficiency and $\beta$ thalassaemia minor *The proportion of Haemoglobin A to* $A_2$ *would not be* changed in the latter condition when there is a co-existent iron deficiency nor would Haemoglobin F if it is present, disappear Difficulties arise in this case, not because of the disappearance of the diagnostic fraction, but because an accepted criterion for the diagnosis of $\beta$ thalassaemia minor is a normal or high serum iron together with a hypochromic blood picture.

Electrophoresis can illustrate the fast moving fractions Haemoglobin H ($\beta_4$) and Barts ($\gamma_4$) The presence of Haemoglobin H can be confirmed by a special test for red cell inclusion bodies, which form because Haemoglobin H denatures easily within the red cell.

Patients with Haemoglobin H have a large spleen and may well have undergone a previous splenectomy In this case the red cells present large inclusion bodies and peculiar deposits, which can be seen without any treatment of the blood. The spleen is thought to be capable of actually removing the intracellular precipitate

from the red cells as they pass through that organ. Certainly when the spleen is removed a very bizarre blood picture results and allows instant diagnosis.

*Treatment*

$\alpha$ Thalassaemia major is incompatible with life and $\alpha$ thalassaemia minor causes no clinical disability. One can therefore confine oneself to the treatment of $\beta$ thalassaemia major and minor and Haemoglobin H disease.

*$\beta$ Thalassaemia Major*

The attitude to the treatment of these cases will be somewhat similar to the attitude of the physician treating children with leukaemia. Such children will need constant attention because the longer the disease lasts the more often do they need transfusion. If it is possible to nurse them through to the age of 10 or 11 there may be a slight improvement and there may be longer intervals between the times of transfusion. Although the condition is so hopeless it is still important to give constant attention to parents and family otherwise the parents in their despair may turn to a quack who may ruin them financially. It is also important to remind the parents, if they have other children, that their attention should not be entirely concentrated on this child with $\beta$ thalassaemia major. There may be children with $\beta$ thalassaemia minor who themselves may need a fair amount of medical attention. Also the psychological injury to children neglected for the sake of a single sibling should be avoided with judicious counsel by the medical attendant.

In order to avoid haemochromatosis, transfusion should be kept to a minimum and iron therapy avoided. Routine folic acid should be given and infections vigorously treated. In thalassaemia major splenectomy may result in a transient improvement in the clinical state but it is important to warn the parents that this does not represent a cure.

*$\beta$ Thalassaemia Minor*

The treatment of $\beta$ thalassaemia minor consists in vigorously treating secondary stresses, such as infection, and in supporting the increased red cell production as a rule but especially during

pregnancy with a small maintenance dose of folic acid and a good protein diet. Blood transfusions should always be given warily and only when clinically essential, again because of the danger of haemochromatosis. It is particularly wrong to give patients iron because the hypochromia is not due to iron deficiency The excessive erythropoiesis in the over active marrow itself stimulates the bowel to over-absorb iron. The over-absorption of iron causes its deposition in the tissues and the situation is only made worse with blood transfusion. Indeed, in thalassaemia major the cause of death very often is heart failure due to haemochromatosis of the cardiac muscle. Haemochromatosis may even develop without iron therapy We have seen two British adults initially diagnosed as haemochromatosis, who had never had any iron therapy but who were subsequently found to belong to the small group of native British thalassaemics.

One must remember that it is not altogether rare for multiparous women *with β thalassaemia minor also to suffer from a deficiency* of iron. Because of the pale red cells due to the thalassaemic process this is not easy to recognise by a simple examination of the blood film. One would be suspicious of a complication if the haemoglobin level of the patient dropped below the figure of about 10 g per 100 ml. If there was a severe co-existent iron deficiency the mean corpuscular haemoglobin concentration would be considerably reduced and the serum iron would be low Finally the iron stores could be assessed by staining some marrow fragments with the Prussian blue reaction. In all cases the clinician must prove iron deficiency before he embarks on iron therapy Patients with the heterozygous state for β thalassaemia often lead perfectly normal active lives, the haemolytic process being usually of the compensated type. It should be ensured that this compensation remains adequate by protecting the erythropoietic system undue additional stresses. At the same time one does not wish to turn these patients into chronic invalids. Many millions of people have the thalassaemia trait and survive without undue difficulty even under the most primitive conditions.

### *Haemoglobin H Disease*

Patients with Haemoglobin H disease are on the whole more severely afflicted than patients with thalassaemia minor The treatment of Haemoglobin H disease is in every way similar to that of thalassaemia minor which has been outlined above. However be-

cause of its greater severity a case of Haemoglobin H disease should remain under medical supervision and receive permanent folic acid therapy

Interaction between Thalassaemia and Abnormal Haemoglobins

α Thalassaemia only depresses the production of *normal* α chains and β thalassaemia only depresses the production of *normal* β chains. *In double heterozygotes for the β thalassaemia gene*, and for a gene for the common abnormal β chain variants of Haemoglobin A, such as Haemoglobins S C, D (Los Angeles or Punjab) and E, it is noteable that only the formation of Haemoglobin A is affected and that the β thalassaemia gene does not interfere with the production of abnormal β chains in the non A haemoglobin. For example, there are thus two types of haemoglobin distribution in the sickle cell trait carrier the ordinary sickle-cell trait carrier possesses more Haemoglobin A than S but if he is also a heterozygote for the β thalassaemia gene, the proportion is reversed and Haemoglobin S is the preponderant adult pigment. This is because the production of Haemoglobin A with its normal β chain is selectivity depressed by the β thalassaemia gene. In other words, interaction between β thalassaemia and an abnormal β chain haemoglobin is due to the fact that β thalassaemia specifically interferes with only one of the two haemoglobins present, that which contains the normal β chain.

From this it becomes clear that β thalassaemia will not interact if the two haemoglobins are Haemoglobin A and an abnormal α chain haemoglobin. The abnormal α chain haemoglobin carries the same normal β chain as Haemoglobin A. It follows that β thalassaemia cannot interact in such a carrier of an abnormal α chain haemoglobin and there will therefore be no difference in the proportion of A to the abnormal α chain haemoglobin whether or not β thalassaemia is also present.

All the common variants of adult haemoglobin are in fact β chain variants. Persons carrying one of the rare α chain abnormalities such as Haemoglobin J α, together with thalassaemia minor will have the typical blood picture of thalassaemia minor They will show no interaction of the proportions of the two haemoglobins, which is the same as that seen in the ordinary heterozygote for Haemoglobin J α—Haemoglobin J α will be about 30 % of the total pigment present

Just as $\beta$ thalassaemia interacts with abnormal $\beta$ chain variants, so does $\alpha$ thalassaemia interact with abnormal $\alpha$ chain variants. When a gene for $\alpha$ thalassaemia is inherited together with the heterozygous state for an abnormal $\alpha$ chain variant, this abnormal haemoglobin will be produced in preference presumably because the $\alpha$ chain thalassaemia only depresses the production of normal $\alpha$ chains. If one of the patient's genes for $\alpha$ chain production is depressed by $\alpha$ thalassaemia and the other gene is producing an abnormal $\alpha$ chain haemoglobin, the patient does not possess the one normal production line for $\alpha$ chains of the ordinary $\alpha$ chain thalassaemia heterozygote, which can do the work of two Thus, this double heterozygote for an abnormal $\alpha$ chain haemoglobin and $\alpha$ thalassaemia forms no or only very little Haemoglobin A and in infancy besides the abnormal $\alpha$ chain haemoglobin, Haemoglobin Barts ($\gamma_4$) is found and in the adult Haemoglobin H ($\beta_4$) Two such cases of interaction have been described involving the rare $\alpha$ chain haemoglobin variants I and Q $\alpha$ chain thalassaemia has also been found combined with the heterozygous state for Haemoglobins S and C. Here, no interaction occurs because S and C are abnormal $\beta$ chain variants, and the $\alpha$ thalassaemia gene would affect equally the normal and the abnormal haemoglobin because both S and C contain normal $\alpha$ chains. The proportion of Haemoglobin A to S and Haemoglobin A to C is the same as in the uncomplicated AS or AC heterozygote—the abnormal haemoglobin being about 40% of the total pigment.

The clinical picture may be used as a guide, but the last resort is always a family study

*Summary*

Whereas the haemoglobinopathies arising from abnormal haemoglobins are the outcome of the replacement of Haemoglobin A by genetically determined abnormal haemoglobin, the thalassaemias arise from genetically determined disability of Haemoglobin A production. Haemoglobin A consists of alpha and beta chains, and Thalassaemia may affect either of the two alpha- and beta-Thalassaemia. The most severe condition is homozygous alpha Thalassaemia. On the other hand, the heterozygous alpha Thalassaemia is perhaps the least severe of all Thalassaemias. If, in addition to heterozygous alpha Thalassaemia, gene for H disease is present, Haemoglobin H disease results. The homozygous form of beta Thalassaemia rarely permits survival to adult age The heterozygous beta Thalassaemia condition can vary from clinically severe to an almost unnoticed state The production of polypeptide chains passes many

stages, and Thalassaemia can arise from a genetically determined defect at any of these steps, at the regulator genes, structural genes, the messenger RNA, and at the final production of the polypeptide chain on the ribosome from activated amino-acids in the presence of the messenger RNA.

## *Zusammenfassung*

Während die durch abnorme Hämoglobine bedingten Hämoglobinopathien das Ergebnis eines Ersatzes von HbA durch ein genetisch determiniertes abnormes Hämoglobin sind, entstehen die Thalassämien durch ein genetisch determiniertes Unvermögen zur Bildung von HbA. HbA besteht aus α- und β-Ketten, und die Thalassämie kann jede der beiden Ketten betreffen. α- und β-Thalassämie. Am schwersten ist die homozygote α-Thalassämie. Andererseits ist die heterozygote α-Thalassämie vielleicht die leichteste Form aller Thalassämien. Wenn neben der heterozygoten α-Thalassämie ein Gen der HbH-Krankheit vorliegt, ergibt sich eine HbH Krankheit. Die homozygote Form der β-Thalassämie erlaubt selten ein Überleben bis ins Erwachsenenalter. Die heterozygote β-Thalassämie kann variieren von einer klinisch schweren bis zu einer beinahe unerkannten Form. Die Bildung von Polypeptidketten durchläuft viele Stadien, und in jedem derselben kann eine Thalassämie durch einen genetisch bedingten Defekt hervorgerufen werden an den Regulator-Genen, den Struktur-Genen, der Überträger RNA und an der zuletzt erfolgenden Produktion der Polypeptidkette am Ribosom aus aktivierten Aminosäuren in Gegenwart von Überträger RNA.

## *Résumé*

Pendant que les hémoglobinopathies provenant d'hémoglobines anormales sont le résultat du remplacement de l'hémoglobine A par une hémoglobine anormale déterminée génétiquement, les thalassémies proviennent d'une incapacité déterminée génétiquement de produire de l'hémoglobine A. L'hémoglobine A se compose de chaînes alpha et beta, et dans la thalassémie les unes ou les autres sont affectées alpha- et beta-thalassémie. La forme la plus grave est la thalassémie alpha homocygote. De l'autre côté, la thalassémie alpha hétérocygote est peut-être la moins grave de toutes les thalassémies. Si en plus de la thalassémie alpha hétérocygote un gène pour la maladie H est présent, il en résulte une maladie d'hémoglobine H. La forme homocygote de la thalassémie beta ne permet que rarement une survie jusqu'à l'âge adulte. La thalassémie hétérocygote beta peut varier d'une forme clinique grav à un état à peine décelable. La production de chaîne de polypeptides passe par plusieurs stades, et la thalassémie peut apparaître, venant d'un défaut génétique, à chacun de ces stades au niveau des gènes régulateurs, des gènes structuraux, de l'ARN messager et de la production finale des chaînes de polypeptides sur les ribosomes à partir des acides aminés activés en présence de l'ARN messager

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# Die Reifung des Retikulozyten

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## I. Morphologie

Die Retikulozyten wurden 1865 erstmals von Erb (9) beschrieben und 1891 von P. Ehrlich (8) wiederentdeckt. Letzterer verstand darunter noch nicht voll ausgereifte Erythrozyten, die nach Behandlung mit basischen Farbstoffen eine körnige oder netzartige Struktur erkennen ließen, die sogenannte *substantia reticulo-filamentosa*. Heilmeyer und Westhäuser (14) teilten später die Retikulozyten aufgrund der Form und Intensität dieses Netzwerkes in 4 Reifungsstadien ein: Knäuelform, Netzform, unvollständige Netzform und Körnchenform. Mit diesen morphologischen Kriterien ist eine ungefähre Zellaltersbestimmung möglich. Heute wissen wir, daß die substantia reticulo-filamentosa ein färberisches Kunstprodukt ist. Der basische Farbstoff Brillantkresylblau fällt sowohl die partikulär gebundenen ribosomalen Ribonukleinsäuren als auch die «Boten-RNS» (messenger RNA) aus, dabei werden Konglomerate von mehreren Ribosomen gebildet, deren Eiweißanteil mitpräzipitiert (Coutelle und Rapoport 7 a, b; Rosenthal *et al.*, 40). Das Netzwerk besteht also aus gefällten Ribonukleoproteiden und nicht etwa aus Chromosomen, wie lange Zeit angenommen wurde.

Die Retikulozyten stellen keine originären oder endgültigen Zellformen dar, sie sind Übergangsstadien von den kernhaltigen Erythroblasten zu den ausgereiften Normozyten. Ihre quantitativen Relationen zu den Erythroblasten einerseits und den Erythrozyten andererseits wurden in den letzten Jahren besonders von Leibetseder (20), Weicker (58 a–d), Cronkite *et al.* (6) sowie Lajtha (19 a, b) untersucht.

LEIBETSEDER (20) und WEICKER (58) fanden aufgrund von Zellkernmessungen fünf verschiedene Erythroblastengenerationen deren Zellzahlen im umgekehrten Verhältnis zu ihren Kernvolumina stehen. Die Kernklasse $K_2$ (58 a) entspricht dem Proerythroblasten, $K_1$ dem jungen basophilen Erythroblasten $K_{1/2}$ dem basophil polychromatischen, $K_{1/4}$ dem polychromatischen und $K_{1/8}$ dem oxyphil polychromatischen Erythroblasten (Abb. 1) Aufgrund der Mengenrelation der fünf Erythroblastengenerationen untereinander ($K_2$ = 0,75 $K_1$ = 1,25 $K_{1/2}$ = 2 $K_{1/4}$ = 4 $K_{1/8}$ = 8) nimmt WEICKER (58) eine *kern-homoplastische Teilung* der Proerythroblasten an. Danach soll sich der Proerythroblast sowohl hemiplastisch in einen $K_1$ Erythroblasten als auch homoplastisch in einen anderen Proerythroblasten teilen. Die homoplastische Teilung soll der Bildung eines neuen Erythrons und damit der Erhaltung der Proerythroblasten, die hemiplastische Teilung dagegen der eigentlichen Erythropoese dienen. Es folgen anschließend 3 succedane Reifungsteilungen bis zu den $K_{1/8}$ Erythroblasten, die durch Kernausstoßung zu Retikulozyten werden.

HEMMERLE (15) zeigte nun, daß die einzelnen Teilungsschritte des Erythrons synchron ablaufen. WEICKER (58 c) berechnete für alle 5 Erythroblastenklassen etwa gleich lange Generationszeiten

Abb. 1 Die Kernvolumenverteilung der normalen Erythropoese und das sich aus ihr ableitbare Teilungsgesetz (WEICKER, 58d)

Abb. 2. Das Modell des zeitlichen Ablaufes im Erythron. Erklärung für die Gleichzeitigkeit von Mitosen im Erythroblastennest (Wrucker, 58c).

von 21,5–23 Stunden, aber unterschiedlich lange Mitosezeiten von 2,5 h für die Proerythroblasten und 1,25 h für die Erythroblasten, die Summe von Generations- und Mitosezeit soll also immer 24 h betragen. Daraus ergäbe sich ein brauchbares Zeitmodell für die Erklärung des synchronen Mitoseablaufes im Erythron (Abb. 2)

Die hervorragende Leistungsfähigkeit der Erythropoese läßt sich nach Wrucker (58 d) durch einen Vergleich der *Zeitmengenprodukte* der verschiedenen Zwischenglieder errechnen (Abb 3)

Durch die 24-stündig aufeinander folgenden Teilungen und die Verlängerung der Lebensspanne des im Normozyten verselbständigten Erythroblastenplasmas auf 120 Tage kommt es zu einer effektiven Potenzierung die einem Wirkungsquotienten von 1 1000 bis 1:2000 entspricht. Das bedeutet, daß ein Proerythroblast in *Tageseinheiten* ausgedrückt scheinbar 1000 bis 2000 Erythrozyten liefert, realiter allerdings nur 8–16. Dabei ist umstritten, ob sich die Retikulozyten noch einmal teilen können, wie Rind (39) im Phasen-

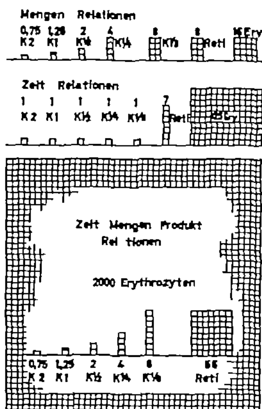



*Abb. 3.* Die Wirkungssteigerung der Erythropoese ausgedrückt durch die Zeit-Mengen-produkte vom Proerythroblasten bis zum Erythrozyten (W[illegible], 38d)

kontrastmikroskop gelegentlich beobachtet haben will. – Unter Zugrundelegung eines Wirkungsquotienten von 1 : 1000 sind nur etwa 240 ml aktives Knochenmark erforderlich, um die bei der normalen Zellmauserung täglich sequestrierten $200 \times 10^9$ Erythrozyten zu ersetzen. Das menschliche Knochenmark hat also mit einem Gesamtvolumen von 3–3,5 l (MECHANIK, 29) enorme Reserven.

LAJTHA (19) postuliert hingegen aufgrund von ferrokinetischen Studien und $H_3$-Thymidin-Markierungen eine pluripotente Stammzelle, deren morphologischer Nachweis allerdings bisher nicht gelingt. Für eine solche Stammzelle spricht, daß durch die humorale Einwirkung von Erythropoetin entstandene Proerythroblasten sich durch eine homoplastische Teilung anscheinend nicht mehr selbst erhalten können, sondern sich unweigerlich zu Erythroblasten

weiterdifferenzieren müssen, also ihre eigene Zahl nicht konstant halten können (Abb 4)

Nach der Differenzierung der Stammzelle *S* zum Proerythroblasten (Pro) folgen nach LAJTHA (196) drei Zellzyklen von je 20-stündiger Dauer bis zum älteren basophilen Erythroblasten (Ia–Ic) Ein Teil der basophilen Erythroblasten soll dabei zugrunde gehen (unwirksame Erythropoese) Es folgt dann ein 25–30-stündiger Zellzyklus des polychromatischen Erythroblasten (II) und ein 30–50-stündiger Zyklus des oxyphil polychromatischen Normoblasten (III) aus denen dann die Mark Retikulozyten entstehen (IV) Ein Teil der polychromatischen Erythroblasten (II oben) sollen nicht mehr der letzten Teilung unterliegen, sondern direkt ohne Mitose zu oxyphilen Normoblasten und Markretikulozyten (III und IV') heranreifen können.

Abb 4. Das aufgrund von ferrokinetischen Messungen und von $H_3$-Thymidin-Markierungen beruhende Modell des Erythrons. Nähere Erklärungen s. Text (LAJTHA, 196)

Für Lajthas Theorie wird angeführt, daß alle Proerythroblasten schon Hämoglobin synthetisieren und sich irreversibel durch Teilung und Differenzierung zu Erythroblasten bzw. Retikulozyten umwandeln. Zugunsten einer pluripotenten Stammzelle sprechen auch die Untersuchungen von Becker, McCulloch und Till (59) die in der Milz von letal bestrahlten Mäusen *Stammzell-Klone mit* einheitlichem Chromosomen-Karyotyp nachwiesen, die alle 3 Zelllinien des Knochenmarkes bilden konnten. Es bleibt aber noch abzuwarten, welches Erythron-Modell der Wirklichkeit näher kommt.

## *2 Biochemie*

Die Erythroblasten verlieren bei der Reifung nicht nur den Kern, die Retikulozyten nicht nur die Mitochondrien und Mikrosomen, sondern mit diesen Strukturen auch die Fähigkeit zur Synthese von DNS RNS Proteinen, Häm, Lipiden und zur Neubildung von Purin- bzw Pyrimidinbasen aus einfachen Bausteinen. Diese biochemischen Reifungsprozesse sind in der Abb 5 schematisch nach London (25 a) wiedergegeben und sollen einzeln besprochen werden



| | | | |
|---|---|---|---|
| Struktur | | | |
| Kern | + | 0 | 0 |
| Mitochondrien | + | + | 0 |
| Mikrosomen | + | + | 0 |
| Nucleinsäuren-Synthese | | | |
| DNS | + | 0 | 0 |
| RNS | + | + | 0 |
| Protein-Synthese | + | + | 0 |
| Häm-Synthese | + | + | 0 |
| Lipid-Synthese | + | + | 0 |
| K-H-Stoffwechsel | | | |
| Glycolyse | + | + | + |
| Hexosemonophosphat-Zyklus | + | + | + |
| Tricarbonsäure-Zyklus | + | + | 0 |
| Glycerin-1 P-Zyklus | + | 0 | 0 |
| Cytochrom-System | + | + | 0 |

*Abb. 5.* Schematische Darstellung der strukturellen und biochemischen Prozesse bei der Erythroblasten-Reifung (London, 25a).

a) *Die Nukleinsäuren.* Thorell (51 52) wies durch mikrospektrophotometrische Messungen in den Proerythroblasten reichlich DNS und RNS nach und fand eine Zunahme dieser Substanzen während der Generationszeit. Gleichzeitig konnte er eine Proteinsynthese registrieren. Mit zunehmender Reifung der Erythroblasten nahm der Gehalt an DNS RNS und die Proteinsynthese zugunsten der Hämoglobinbildung ab. Obwohl dabei das Zellvolumen sich verringert, bleibt das Produkt Zellvolumen × Zellzahl konstant. Diese Verhältnisse sind in der Abb 6 schematisch nach Stich (50) aufgeführt.

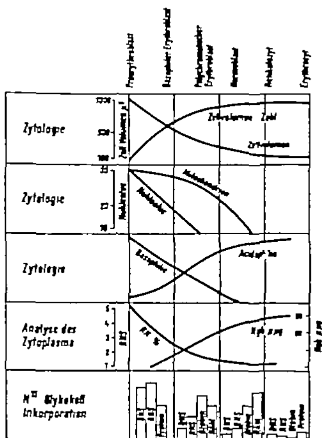


Abb 6. Veränderungen von Zellvolumen, Zellzahl, Kern, Mitochondrien, Anfärbbarkeit, RNS, Hämoglobingehalt und die 15 N-Glyzin-Inkorporation in die Fraktionen von DNS, RNS, Proteine Häm und Globin während der Reifungsprozesse (Stich, 50).

*Abb. 7* Vereinfachtes Modell der Proteinsynthese (Watson, 57b) DNA = Desoxyribonukleinsäuren; RNA = Transfer Ribonukleinsäure m RNA = Boten-Ribonukleinsäure; RNA = Ribosomen-Nukleinsäuren; AA = Aminosäuren, ATP = Adenosin-Triphosphat; AMP = Adenosin-Monophosphat; GTP = Guanosin-Triphosphat.

Was bedeutet nun die Abnahme der DNS- und RNS-Synthese während der Zellreifung? Die DNS ist im Kern lokalisiert und als Träger der gesamten genetischen Information des Erythrons anzusehen. Sie hat ein Molekulargewicht von mehreren Millionen, liegt nach Crick und Watson (5 57) als gefaltete Doppelspirale vor und besteht nach Chargaff und Davidson (4) außer Desoxyribose und Phosphat nur aus vier verschiedenen Basen, deren Reihenfolge die genetische Information beinhaltet. Diese Basen sind die Purine Adenin (A) und Guanidin (G) sowie die Pyrimidine Cytosin (C) und Thymin (T) Bei allen untersuchten Lebewesen ist die Summe der Purinbasen A + G gleich der Summe der Pyrimidinbasen C + T außerdem sind die Quotienten A/T und G/C immer 1 *In vivo* wird nur die Information eines DNS-Stranges folgendermaßen übertragen (Marmur *et al.* 63) Eine DNS-abhängige RNS-Polymerase katalysiert die Synthese einer einsträngigen RNS die komplementär zu einem DNS-Strang ist (Hurwitz *et al.* 62) Die RNS unterscheidet sich von der DNS nicht nur durch den Riboseanteil, sondern auch durch den Ersatz der Pyrimidinbase Thymin (T) durch Uracil (U) Man unterscheidet eine lösliche *Boten-RNS* Fraktion im Nucleolus und Hyaloplasma von einer unlöslichen, partikulär gebundenen Fraktion, die in den Ribosomen lokalisiert ist (Abb. 7)

Die DNS liefert also die Matrize für die «Boten-RNS» (mRNA) welche die genetische Information an die Ribosomen heranbringt und mehrere Ribosomen (mindestens 5) zu Polysomen zusammenfügt (Warner et al., 56 Gierer, 12) Die Ribosomen sind die Stätten der eigentlichen Proteinsynthese, sie besitzen eine Sedimentationkonstante von 78 S und bestehen aus zwei Untereinheiten einer mit «Boten RNS» verbundenen Fraktion mit 30 S und einer 50 S Fraktion, an welcher die wachsende Polypeptidkette fixiert ist. Die Polypeptidketten werden Aminosäure pro Aminosäure aufgebaut mit Hilfe eines spezifischen 3-basischen *Triplet-Code* für jede Aminosäure, wie von Nirenberg und Matthaei gezeigt werden konnte (64) Vor dem Einbau der Aminosäuren in die Polypeptidkette der Ribosomen müssen diese zunächst durch ATP zu Aminosäure AMP aktiviert und dann an eine spezifische niedrig molekulare *Transfer RNS* (s-RNA) gebunden werden (Hoagland et al., 61) Ihr Einbau erfordert als Energiequelle Guanosin Triphosphat (GTP) und als Katalysatoren sog *Transfer Enzyme* Die Rolle von DNS und RNS bei der Proteinsynthese geht aus der Abb. 7 nach Watson hervor (57 b)

Abnahme von DNS und RNS während der Zellreifung bedeutet also Verlust der Eiweißsynthese Die Retikulozyten enthalten noch alle RNS-Fraktionen, die ihnen zwar die Synthese von Hämoglobin, aber anscheinend nicht mehr die Neubildung von Struktur und Enzymproteinen in meßbarer Quantität erlauben, denn deren Synthese ist nach Thorell (52) abgeschlossen, wenn die Hämoglobinisierung ihr Maximum erreicht. Der Verlust von DNS und RNS während der Reifungsvorgänge wird von Brody et al. (3) auf eine Aktivierung der Desoxyribonukleasen und Ribonukleasen zurückgeführt. Retikulozyten können *de novo* noch den Purin aber nicht mehr den Pyrimidinring synthetisieren (Lowy et al., 27)

b) *Die Hämoglobinsynthese* Die Hämoglobin Bildung beginnt schon in den Proerythroblasten und nimmt bis zu den $K_{1/2}$ Erythroblasten ständig zu, während DNS und RNS-Synthese abfallen. Ja selbst der Retikulozyt kann noch Blutfarbstoff bilden (London 25 26 Thorell, 52) Nach Lajtha (19 a) sowie Sondhaus (49) benutzen die Proerythroblasten schon genügend Eisen für die spätere Hämsynthese. Da bei der Retikulozyten-Reifung in Abhängigkeit von der Zellatmung Stromaeiweiß verbrannt wird, nimmt Rapoport (34) an, daß der Globinanteil des Blutfarbstoffes teilweise aus dem beim Stroma Abbau anfallenden Rest-Stickstoff Pool gebildet

wird. Das endogene Substrat der Retikulozyten-Atmung sind nämlich Aminosäuren und nicht Glukose (Brandt und Rapoport 2)

Anscheinend werden die α- und β-Ketten des Globins in den Polysomen unabhängig voneinander synthetisiert, eine Boten RNS hierfür konnte bisher noch nicht isoliert werden (Huehns und Shooter, 16) Da die Retikulozyten noch Hämoglobin, aber keine neue RNS mehr produzieren, muß man folgern, daß die Boten RNS für die Globinsynthese stabiler als die anderer synthetischer Stoffwechselwege ist (Marks et al 28)

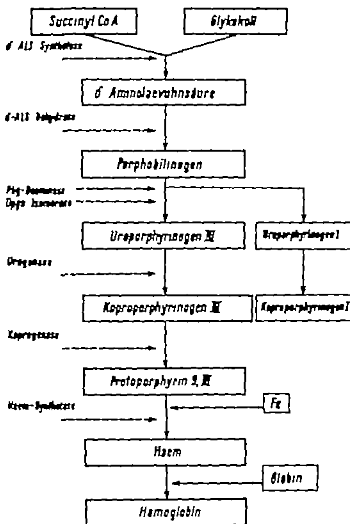



*Abb. 4.* Schematische Darstellung der Häm-Synthese mit den einzelnen Reaktionsschritten (Stich, 50).

Die Häm Bildung findet im Zytoplasma bzw. der erste und die beiden letzten Schritte in den Mitochondrien statt, daher läuft sie noch in den Retikulozyten, aber nicht mehr in den mitochondrienlosen Normozyten ab. Letztere besitzen nach HEILMEYER (13) und GAJDOS (11) alle Enzyme der Häm-Synthese mit Ausnahme der δ-Aminolävulinsäure-Synthetase und Häm-Synthetase (Abb. 8).

Die Kenntnisse der Porphyrin-Strukturen verdanken wir besonders HANS FISCHER (10) und seiner Schule, die einzelnen Schritte der Häm-Biosynthese wurden vor allem von SHEMIN, RITTENBERG und LONDON (47, 48, 26) aufgeklärt, sie sind in der Abb. 8 nach STICH (50) wiedergegeben. Über ihre Regulation ist inzwischen einiges bekannt. Die δ-Aminolävulinsäure-Synthetase wird durch 2 wertige Eisen-Ionen und Pyridoxalphosphat, die folgende Dehydratase durch das Hormon Erythropoetin stimuliert (STICH, 50). Eine weitere Kontrolle der Porphyrinsynthese erfolgt durch einen Rückkopplungs-Mechanismus, da Hämin in 0,1 mM Konzentration die δ-Aminolävulonsäure-Synthetase um 50% hemmt (LONDON, 25 b). Zunahme der Häm-Konzentration in den Erythroblasten und Retikulozyten bedeutet somit Bremsung, Zunahme der Eisenkonzentration aber Stimulierung der Porphyrinbildung, also sinnvolle Vorgänge.

Nach KATZ und JANDL (18) wird das eisentransportierende Transferrin durch spezifische Rezeptoren an die Oberfläche der Erythroblasten bzw. Retikulozyten gebunden, die Eisenaufnahme durch die Zelle erfordert Energie, das Transferrin gibt sein Eisen ab und wird danach vom Rezeptor wieder freigegeben, außerdem kann auch Eisen durch eine der Pinozytose vergleichbare *Rhopheozytose* von Erythroblasten aufgenommen werden (BESSIS und BRETON-GORIUS, 60). Möglicherweise wird das einverleibte Eisen zunächst in den roten Vorstufen an Apoferritin gebunden und dann als Ferritin an die Mitochondrien herangeführt. Die Freisetzung des Eisens aus den Mitochondrien beim Einbau in das Protoporphyrin IX, welcher durch die Häm-Synthtase katalysiert wird, erfordert Pyridoxalphosphat. Diese Beziehungen sind in der Abb. 9 schematisch nach LONDON (25 b) wiedergegeben.

Bei einer mittleren Erythrozyten-Lebensdauer von 120 Tagen müssen durch die Blutmauserung von 200 × $10^9$ Zellen täglich 6,5 g Hämoglobin nachgebildet werden, d.h. 260 mg Häm.

c) *Die Lipidsynthese.* Die Erythroblasten synthetisieren Lipide, weniger die Retikulozyten, in den reifen Normozyten findet keine

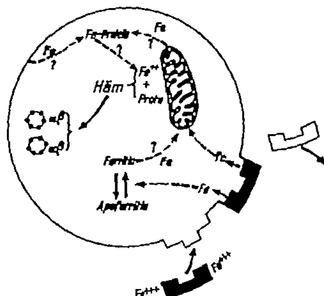



*Abb. 9* Schematische Darstellung der Eisen-Inkorporation und der Synthese von Häm und Globin in den unreifen roten Vorstufen. Der Kern wurde fortgelassen. Ein Molekül Eisen-bindendes Transferrin nähert sich gerade einem spezifischen Rezeptor an der Zellmembran, ein anderes Transferrin-Molekül ist eben an einen Rezeptor gebunden und übergibt sein Eisen an die Zelle. Ein 3. Transferrin-Molekül hat sein Eisen bereits an die Zelle abgegeben und kehrt ins Plasma zurück. Der Transport des Eisens in der Zelle zum Ferritin und Nicht-Häm-Eisenproteide, die Inkorporation von Eisen in die Mitochondrien sowie die Synthese von Häm und Globin sind im Text erläutert (*London*, 25b).

de novo-Fettbildung mehr statt. Nach Thorell (52) sowie Augustin und Rapoport (1) verlieren die Retikulozyten bei der Reifung besonders Phosphatide, aber kaum Cholesterin. Wahrscheinlich stammen diese Phosphatide aus den Zell- und Mitochondrienmembranen. Radebrecht *et al.* (33) fanden einen überwiegenden $^{32}$Phosphateinbau in die Lecithin- und Kephalinfraktion der Retikulozyten, dagegen lediglich in die Phosphatidsäuren der reifen Erythrozyten. Auch fehlt Retikulozyten und Erythrozyten die $\beta$-Hydroxybutyrat Dehydrogenase für den Fettsäureabbau zu Aceto-Acetyl CoA. Löhr, Waller und Karges (22) zeigten, daß Retikulozyten und Normozyten weder eine zytoplasmatische Glyzerin 1 Phosphatdehydrogenase noch eine mitochondriale Glyzerin 1 Phosphat oxydase besitzen, also nicht über den Büchnerschen Glyzerophos-

phat Zyklus verfügen. Dieser Zyklus ist aber in normalen Erythroblasten, fetalen Erythrozyten und Leukozyten vorhanden und persistiert in den $Hb_F$-Zellen bei β-Thalassämie (Löhr und Waller, 24 b)

1 g Erythrozyten enthält 350 mg Hämoglobin, aber nur 5 mg Lipide, davon hauptsächlich Phospholipide (66%) freies Cholesterin (26%) Triglyzeride (3%) sowie 5% Glykolipide. 95% aller Lipide sind im Stroma lokalisiert (Ways und Reed 65)

Wenn auch die Plasmalipide teilweise mit den Erythrozytenmembran Lipiden ausgetauscht werden, ist es dennoch ein Wunder daß trotz fehlender Lipidsynthese die Erythrozytenmembranen 120 Tage im Kreislauf überleben können.

d) *Der Kohlenhydratstoffwechsel* Bei der Zellreifung treten erhebliche Verschiebungen im energieliefernden Kohlenhydratstoffwechsel ein Während die Retikulozyten einen vorwiegend aeroben Metabolismus aufweisen – wobei als endogenes Substrat hauptsächlich Aminosäuren verbrannt werden und die Zuckerverbrennung nur 10–15% ausmacht (Brandt und Rapoport 2) Scheuch *et al.* (44) – verlieren die Erythrozyten bei ihrer Reifung mit den Mitochondrien-Strukturen die Enzyme der Atmungskette, deshalb ist ihr Stoffwechsel anaerob.

Retikulozyten bauen 75% der Glukose oxydativ über den Pentosephosphatzyklus und nur 25% über den glykolytischen Embden Meyerhof Weg ab (Rapoport *et al.*, 37) reife Normozyten dagegen nur 11% über den Pentosephosphatzyklus und 89% über die Glykolyse (Murphy 30) Diese Umschaltung vom vorwiegenden Pentosephosphatzyklus der Retikulozyten auf die Glykolyse der Erythrozyten kommt durch das Co-Enzym II Nikotinsäureamid-dinucleotid phosphat (NADP) zustande, das wegen der fehlenden Atmungskette in den Normozyten nicht mehr ausreichend reoxydiert werden kann. NADP ist somit der limitierende Faktor des Pentosephosphatzyklus, wie Löhr und Waller (23 b, 54) sowie Rapoport *et al.* (37) nachwiesen.

Ausführliche Enzymuntersuchungen an Retikulozyten und Normozyten von Kaninchen (Rapoport 34) sowie von Menschen (Löhr und Waller, 23 a, b 24 Schabert 42) ergaben – berechnet auf das Zellvolumen – folgende Ergebnisse bei der Retikulozytenreifung

Fast völliges Schwinden der Zellatmung des Atmungskettenenzyms Zytochrom-Oxydase, sowie der Zitratzyklusfermente Suc

cino-dehydrogenase und Akonitase während Malat-dehydrogenase (MDH) und Isocitrat-dehydrogenase (ICDH) etwa die halbe Aktivität verlieren wie auch RUBINSTEIN *et al.* (41) mitteilten.

*Gleichzeitig fallen die glykolytischen Durchflußraten ab* bedingt durch die starke Abnahme der Hexokinase-Aktivität, die für den EMBDEN-MEYERHOF Weg in allen Blutzellen das begrenzende Enzym darstellt (GRIGNANI und LÖHR, 23b KASHKET *et al.* 17

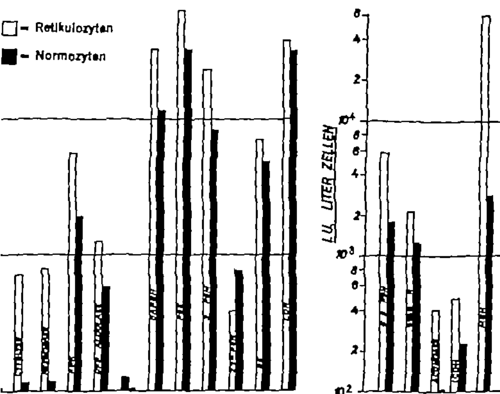



*Abb. 16a und b* Das Verhalten von zahlreichen Enzymaktivitäten berechnet pro 1 Zellvolumen bei der Reifung der Retikulozyten zu Normozyten. Die wiedergegebenen Ergebnisse beruhen auf den Messungen und Untersuchungen von LÖHR und WALLER (23a, b; 24) sowie von RAPOPORT (34) und RUBINSTEIN *et al.* (41)

*Abkürzungen.* FPK = Fruktose-6-Phosphatkinase DFP = Aldolase = Diphosphofruktose-Aldolase; GDH = Glycerin-1 Phosphatdehydrogenase GAPDH = Glycerinaldehyd-3-Phosphat Dehydrogenase; PGK = 3-Phosphoglycerat-1-Kinase; 3-PGM = 3-Phosphoglycerat-Mutase; 2,3-PGM = 2,3-Diphosphoglycerat-Mutase· PK = Pyruvatkinase; LDH = Laktatdehydrogenase G-6-PDH = Glukose-6-Phosphat Dehydrogenase; GSSG-R = Glutathion-Reduktase (NADPH-abh.) ICDH = Isocitrat-Dehydrogenase· MDH = Malatdehydrogenase SUCDH = Succinat-Dehydrogenase; GOT = Glutamat-Oxalacetat-Transaminase.

Ockel, Rapoport *et al.*, 32) Zwar werden auch noch zahlreiche andere Fermente bei der Retikulozytenreifung inaktiviert, so besonders die Glukose-6-phosphatdehydrogenase (G-6-PDH) die Glutamat-oxalacetat transaminase (GOT) die Glutaminase, die Adenosintriphosphatase und Pyrophosphatase deren Inaktivierung ist aber nicht so wesentlich, weil diese Fermente gegenüber der Hexokinase und den Atmungskettenenzymen im großen Überschuß vorliegen (beachte den semilogarithmischen Maßstab auf der Abb 10!)

Eine Kette ist ja bekanntlich nur so stark wie ihr schwächstes Glied. Andere Enzyme wie Laktatdehydrogenase (LDH) Glyzerinaldehyd 3-phosphatdehydrogenase (GAPDH) ändern sich bei der Reifung kaum, die 2,3-Diphosphoglyzeromutase Aktivität nimmt sogar zu (Rapoport 34)

Aus den verschiedenen Verhalten der Enzyme darf man folgende Schlüsse ziehen

1 Die biochemischen Änderungen bei der Retikulozytenreifung sind selektiv und keine allgemeinen statistischen Denaturierungsprozesse der Enzymproteine.

2 Es werden vorwiegend einige Schlüsselenzyme des Kohlenhydratstoffwechsels inaktiviert, besonders die Atmungsfermente, die glykolyse-limitierende Hexokinase und die Glukose-6-phosphat dehydrogenase, das Starterenzym des Pentosephosphatzyklus.

3 Betroffen werden vor allem SH-gruppenhaltige Enzyme sowie Häminfermente.

4 Die Aktivitätszunahme der 2,3-Diphosphoglyzeromutase spricht für eine wachsende Bedeutung des 2,3-Diphosphoglyzerat zyklus in den reifen Erythrozyten, der von Rapoport und Luebering (36) entdeckt wurde. Raderecht *et al.* (33) zeigten in Erythrozyten einen erhöhten Umsatz über diesen Seitenweg der Glykolyse.

Diese biochemischen Veränderungen treten bei der Retikulozytenreifung viel schneller auf, als man aufgrund der biologischen Halbwertszeiten der Erythrozyten-Enzymproteine erwarten kann. Diese betragen z.B für die GAPDH 34 Tage, für die G-6-PDH 53 Tage und für die LDH 165 Tage (Löhr, Waller, Schlegel *et al.*, 24) Die überraschende Geschwindigkeit der Enzyminaktivierungen spricht für einen aktiven gezielten Reifungsprozeß, wie er auch tatsächlich nachgewiesen werden konnte. Rapoport und Gerischer Mothes (35 a-d) wiesen im löslichen Retikulozyten

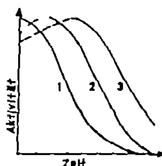



*Abb. 11* Schematische Darstellung der Abnahme der Atmung und des Verlaufes der damit verknüpften Prozesse. Willkürliche Ordinaten. Die unterbrochenen Linien entsprechen der Annahme, daß Hemmstoff und Inaktivator in bestimmten Phasen des Reifungsvorganges zunehmen. 1 = Atmung; 2 = Hemmstoff; 3 = Hemmstoff-Inaktivator (RAPOPORT und GERISCHER-MOTHES, 35)

überstand einen spezifischen Hemmstoff der mitochondrialen Eisen-Flavoproteide Succinat-dehydrogenase und Zytochromreduktase nach. Dieser *R Ü Hemmstoff* ist selbst ein Flavoproteid. Er kann wahrscheinlich erst dann wirken, wenn er in die bei der Reifung veränderten Mitochondrien einzudringen vermag. Da reife Erythrozyten weder Atmungskettenenzyme noch den *R Ü Hemmstoff* enthalten, muß der Hemmstoff selbst wieder inaktiviert werden. Dies geschieht wahrscheinlich durch Freisetzung von Kolamin-Kephalin aus der Retikulozytenmembran. Die zeitliche Reihenfolge von Atmung, Hemmstoff und Hemmstoff-Inaktivator geht aus der Abb. 11 nach RAPOPORT und GERISCHER-MOTHES hervor (35).

Demnach wird zunächst die Retikulozyten-Atmung (1) durch den R. Ü Hemmstoff (2) unterdrückt, dann letzterer durch einen Inaktivator (3) abgebaut.

Daneben fanden die gleichen Autoren noch einen spezifischen Hemmstoff der Zytochrom-Oxydase – des WARBURGschen Atmungsfermentes –, der nur unter aeroben Bedingungen wirkt. Somit ist also der Atmungsverlust bei der Retikulozytenreifung ein gezielter aktiver Prozeß, der durch zwei Hemmstoffe hervorgerufen wird. –

Der Mechanismus der raschen Inaktivierung der Hexokinase, G-6-PDH und anderer SH-Enzyme kann noch nicht zufriedenstellend erklärt werden.

Bekanntlich sind SH-Gruppen sehr empfindlich gegen Oxydationsprozesse, ihre Intaktheit hängt unter anderem vom Redoxsystem des Glutathions ab. Nach SCHRUCH und RAPOPORT (43)

werden zahlreiche SH Enzyme durch eine Zunahme der intra zellulären oxydierten Glutathion-Konzentration zunächst reversibel dadurch gehemmt, daß sich gemischte Disulfide des Enzyms mit dem Glutathion bilden. Die Reversibilität ist zeitabhängig und kann innerhalb von wenigen Minuten durch Zugabe von reduziertem Glutathion (GSH) System etc. erreicht werden. Bei längerem Bestehen der gemischten Enzym-Glutathion-Disulfide kommt es zu einer irreversiblen Inaktivierung der Enzyme. Retikulozyten enthalten nun mehr reduziertes Glutathion als Normozyten, es ist jedoch noch unsicher ob dieser Unterschied für die Inaktivierung der SH Enzyme ausreicht.

### 3 *Die Erythrozyten-Alterung*

Im Anschluß an die Retikulozytenreifung muß noch die Erythrozytenalterung besprochen werden, da beide Prozesse in ein ander übergehen. Reifung bedeutet Differenzierung und Spezialisierung Alterung aber Zelluntergang

Teleologisch gesehen hat der reife Erythrocyt nur die Aufgabe, den Sauerstofftransport zu gewährleisten. Folglich dient sein «Primitivstoffwechsel» vorwiegend der Erhaltung des Hämoglobins in funktionsfähigem Zustande. Immerhin bringt es der ausgereifte Normozyt ohne Kern, Mitochondrien und Mikrosomen, ohne Synthesemöglichkeiten für DNS RNS Proteine, Haem und Lipide sowie ohne nennenswerte Atmungsprozesse noch fertig 120 Tage in der Zirkulation zu leben, ein Vorgang, den wir noch nicht völlig begreifen können! Welche biochemischen Vorgänge führen nun zum Erythrozyten-Untergang? Man muß bei allen Deutungen klar zwischen den physiologischen intravitalen Alterungsprozessen und den unphysiologischen Bedingungen bei der Blutkonservierung unterscheiden. Durch Direkt Übertragung von Spenderblut der Gruppe 0 auf Empfänger der Gruppe A können zu jedem beliebigen Zeitpunkt Spendererythrozyten mit bekanntem Alter aus dem Empfängerkreislauf zurückgewonnen und durch kombinierte Antiseren und Phytagglunitation getrennt werden. Dieses Verfahren ist den anderen Methoden der Zellfraktionierung durch Differentialzentrifugation oder durch abgestufte osmotische Hämolyse überlegen. Mit dieser optimalen Methodik haben Löhr, Waller und Schlegel (24) das Verhalten von über 20 Erythrozytenenzymen in Abhängigkeit vom Zellalter quantitativ untersucht und folgende Ergebnisse erhalten

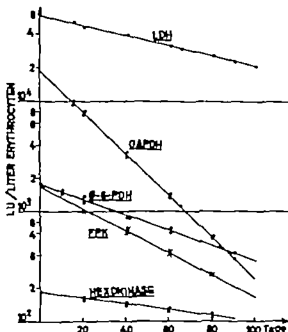



*Abb. 12.* Verhalten von einigen Enzymen während der intravitalen Erythrozytenalterung. Frischblut von Spendern der Gruppe O rh wurde direkt auf Empfänger der Gruppe $A_1$ Rh übertragen. Nach 20–86 Tagen nach der Bluttransfusion wurde das Spenderblut, dessen durchschnittliches Zellalter somit bekannt war, durch Antiseren-Agglutination und anschließende Phytagglutination der Empfänger-Erythrozyten zurückgewonnen, isoliert und daran dann biochemische Untersuchungen vorgenommen. Ordinate: Enzymaktivitäten in Internationalen Einheiten/l Erythrozyten (log. Maßstab). Abszisse: Zeit in Tagen nach den Bluttransfusionen (Löhr, Waller und Schlegel, 23, 24 sowie Schabert 42)

Abkürzungen. LDH = Laktat-Dehydrogenase. GAPDH = Glyzerinaldehyd-3-Phosphat-dehydrogenase. G-6-PDH = Glukose-6-Phosphat-Dehydrogenase. FPK = Fruktose-6-Phosphat Kinase.

Nur einige Schlüsselenzyme werden selektiv rasch inaktiviert. Die Hexokinase, die Fruktose-6-phosphatkinase (FPK), die Glyzerinaldehyd-3-phosphatdehydrogenase (GAPDH) und die Glukose-6-phosphatdehydrogenase (GAPDH). Es handelt sich auch hier wieder um SH-Enzyme. Als Vertreter der langsam inaktivierten Fermente sind hier die Laktatdehydrogenase (LDH) und Methämoglobinreduktase mitangeführt. Der Maßstab ist semilogarithmisch, bei numerischer Auftragung wäre der Aktivitätsabfall exponential. Limitierend für den Erythrozytenstoffwechsel ist auch bei der Alterung die Hexokinase, das Schrittmacher-Enzym der Glykolyse. (Löhr und Waller, 23b. Schabert 42. Ockel,

RAPOPORT et al., 32) Ihre Inaktivierung führt vom 60. Tag der Erythrozytenalterung an zu einem langsam progredierten Zusammenbruch des Energiestoffwechsels mit Abfall von ATP und Anstieg von ADP, auch fällt die intrazelluläre Konzentration des Co-Enzyms I Nikotinsäureamid-Dinucleotid (NAAD) zu diesem Zeitpunkt ab, so daß nicht mehr genügend $NADH_2$ für die Methämoglobinreduktion zur Verfügung steht, was an einem Anstieg des Methämoglobins auf etwa 8% in 80 Tage alten Erythrozyten zu erkennen ist (WALLER, MÜLLER, SCHLEGEL, LÖHR, 55, 54).

Wahrscheinlich ist die Hämolyse der gealterten Blutzellen auf den Mangel an energiereichem Phosphat (ATP) zurückzuführen, das für die Aufrechterhaltung der Zellstruktur nach NAKAO et al. (31) direkt verantwortlich sein soll.

## IV. Schlußbetrachtung

Trotz der großen Detailkenntnis von zahlreichen strukturellen und biochemischen Veränderungen bei der Reifung der Erythroblasten über die Retikulozyten zu den Normozyten ist das übergeordnete Regulationsprinzip für diese Vorgänge noch völlig unbekannt. Sicherlich ist es in der genetischen Information der DNS der Stammzellen determiniert. Das fein abgestimmte Zusammenspiel vieler Strukturgene wird durch übergeordnete Operatorgene gesteuert werden müssen. Eine hormonelle Steuerung – etwa durch das kobalthaltige Glykoproteid Erythropoetin – ist zwar möglich, aber noch nicht genügend aufgeklärt. Dieses Hormon greift stimulierend an der delta-Aminolaevulinsäure-dehydratase an und fördert somit die Hämsynthese, in die wieder Eisen, Globin, Häm und Vitamine regulierend eingreifen. Damit können jedoch längst nicht alle Reifungs- und Alterungsvorgänge erklärt werden. Aus diesen Gründen mußte dieses Referat einen mehr deskriptiven als kausalen Charakter haben.


## Zusammenfassung

Retikulozyten sind kernlose Übergangsformen der Erythropoese, sie haben keine DNS-Synthese mehr. Mittels ihrer RNS sind sie noch zur Synthese von Hämoglobin befähigt, die Mitochondrien gestatten noch mit ihren Atmungskettenenzymen einen oxydativen Stoffwechsel, in welchem vorwiegend Aminosäuren als endogenes Substrat verbraucht werden. Der Glukoseabbau läuft hauptsächlich über den Pentosephosphatzyklus und die Atmungskette, der Fettstoffwechsel ist intakt.

Bei der Retikulozytenreifung werden durch RNasen die lösliche und ribosomale RNS abgebaut, wodurch jede Protein- und Hämoglobinsynthese unmöglich wird. Zwei differente Atmungshemmstoffe führen in den Mitochondrien zur Unterbrechung der

Zellatmung, gleichzeitig tritt eine Inaktivierung einiger SH-Enzyme, besonders der Hexokinase ein. Der reife Normozyt weist einen vorwiegend glykolytischen Stoffwechsel auf, dessen Energieausbeute sehr gering ist. Bei der physiologischen Zellalterung wird durch Inaktivierung der Hexokinase auch die Glykolyse unterbrochen, der ATP-Gehalt fällt ab und ist wahrscheinlich für die Aufrechterhaltung der Zellstruktur nicht mehr ausreichend.

## *Summary*

Reticulocytes are anuclear transition forms in erythropoiesis, with no possibility of synthesizing DNA. They are capable of synthesizing haemoglobin by means of their RNA, and their mitochondria, with respiratory enzymes, permit oxidative metabolism utilizing principally amino acids as endogenous substrate. Glycolysis proceeds mainly by way of the pentose phosphate pathway and the respiratory chain; fat metabolism is intact.

As the reticulocytes, the soluble and ribosomal RNAs are broken down by ribonucleases, preventing any further protein and haemoglobin synthesis. Two distinct respiratory inhibitors interrupt cellular respiration in the mitochondria and at the same time some SH enzymes, especially hexokinase are inactivated. The mature normocyte presents a predominantly glycolytic metabolism, which supplies very little energy. With physiological aging of the cell, inactivation of hexokinase leads also to failure of glycolysis, the ATP content falls and is evidently no longer sufficient to maintain the cell structure.

## *Résumé*

Les réticulocytes sont des formes transitoires sans noyau de l'érythropoïèse, ils n'ont plus de synthèse d'ADN. Grâce à leur ARN ils sont en état de synthétiser de l'hémoglobine, les mitochondries rendent encore possible un métabolisme oxydatif grâce à leurs ferments respiratoires, métabolisme dans lequel les acides aminés servent surtout de substrat endogène. La dégradation du glucose se fait principalement par le cycle du pentose-phosphate et de la chaîne respiratoire, le métabolisme des graisses est intact. Lors de la maturation des réticulocytes, des ribonucléases dégradent les ARN solubles et ribosomiques, toute synthèse de protéines et d'hémoglobines devenant ainsi impossible. Deux substances inhibitrices différentes de la respiration interrompent la respiration cellulaire dans les mitochondries, en même temps les enzymes-SH sont inactivés, en particulier l'hexokinase. Le normocyte mature a un métabolisme avant tout glycolytique dont le rendement énergétique est très faible. Lors du vieillissement physiologique des cellules, la glycolyse est également interrompue par l'inactivation de l'hexokinase, le contenu en ATP diminue et n'est probablement plus suffisant pour maintenir la structure cellulaire.

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# Contribution of Cytology to the Study of Lymphopathies

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In 1934 (20) we proposed the lymph node puncture as a contribution to the study of lymphopathies this work was essentially based on a cytological study of impromtus, for which we chose the May-Grünwald-Giemsa panoptic stain this method permitted quick and precise diagnoses, successive punctures allowing frequent check-ups.

In the years that followed, the cytological study of lymph node material, was greatly substantiated by the development of new techniques and scientific disciplines, with special reference to the contribution of cytochemistry biochemistry cytoimmunology enzymology RNA and DNA dosage, karyotype determination, tissue cultures and finally electron microscopy

From the very beginning the use of specialized cytology had become an important addition to the common histological methods, permitting the classification of lymphopathies with more accuracy Later on, with the advent of the electron microscope, new structural details were added, such as mitochondrial and nucleoli alterations, presence of fibrinoid bodies, vacuoles, possible viruses, etc. the exact role of which has not yet been definitely elucidated.

In this report, we intend to present a synthesis of the contribution of these diverse techniques to the study of lymphopathies in our Institute

## *Interpretation of the Cytological Image of a Lymph Node*

Upon studying the cytological image of a lymph node four different phases have to be considered proliferation, maturation, blastomatous and liberation phases.

*Proliferative Phases*

This phase conditioned by different etiologies, is responsible for a picture of simple hyperplasia, characterized by an equitative distribution of lymphoid cells in different periods of maturation hemocytoblasts, lymphoblasts, prolymphocytes, lymphocytes and plasma cells, as well as cells in mitosis.

This image of evolutive hyperplasia may be brief (days or hours) or may persist for years, depending both on the etiological agent and the response of the organism. Thus, leprosy and syphilis are capable of maintaining for long periods, the same image of proliferative activity in the lymph nodes. On the other hand, an infection due to Gram positive germs, can produce a similar picture, but of very short duration, preceding the leucocytic transformation. Tuberculosis also produces a similar image, leading eventually to fibrosis, including the typical epithelioid cells, or to caseous degeneration.

A reaction brought about by a definite immunological mechanism, as experimentally observed in the rabbit, can also give this image, including very immature cells, held responsible by some investigators of the development of the immunological mechanism (immunoblast)

With this brief enumeration we have tried to point out that a similar picture from the cytological point of view may lead to very different morbid manifestations, depending on the etiopathogenic mechanism at play A neat clinical examination, an etiological search keeping in mind the different immunological mechanisms, will help towards a better interpretation. Furthermore, sometimes the picture of evolutive hyperplasia is of such proliferative intensity that even the most expert specialist may wonder if he is not in the presence of a blastomatous process. In these doubtful cases the karyotype determination the observation of the different evolutive capacity of the lymph node in tissue cultures and the biochemical and cytochemical studies may be useful

Special emphasis must be put on the presence of certain hyper basophilic immature cells, the immunoblasts, as defined by DAMESHEK (12) In spite of the indifferentiation of these cells and their intimate relation with RNA synthesis, they present metabolic differences when compared with other cells considered of malignant nature such as the STERNBERG cell In fact, their staining affinity

for Methylene Blue, points to definite nucleoli differences. Diversities in glycogen content of immature cells of the lymphoid serie and of leukemic lymphoblasts may be observed.

Phase Contrast allows a clear visualization of some structural alterations of the different cell types, while the electron microscope permits the observation of many differential characteristics.

*Maturation or Differentiation Phase*

These images are characterized by a uniform hyperplasia of a definite cell type—prolymphocyte, lymphoblast or hemocytoblast—which does not reach maturation. A hypothetical factor could be at play the presence or absence of which would be responsible for this lack of cellular maturation, having a different meaning according to the degree of differentiation logically the more immature the cells, the more severe the prognosis. The etiological agent remains undetermined.

*Blastomatous Transformation*

We have pointed out that even in images of simple evolutive hyperplasia, embryonal cells may be observed, raising doubts as to whether they are normal immature cells or cells which have undergone blastomatous changes. Up to now in spite of the various techniques used, it has been impossible to differentiate them. Nevertheless, the presence of true cellular monstrosities in cytoplasm, nucleus and nucleoli, may at times permit the characterization of the malignant cell, from the morphological point of view

The contribution of the electron microscope is of great importance. Although many of the observed details have not yet been sufficiently evaluated, it opens a door to new fields of exploration.

The following observations have been carried out in collaboration with Dr VASQUEZ (21–27)

*Nucleoplasmic Lesions*

In general the lymphomatous cell shows an irregular distribution of the chromatin, forming accumulations of different form and size These include variable quantities of osmiophilic granules, disseminated or forming groups although they may be found in the normal cell, their number is exaggerated in the lymphomatous cell.

Another type of nuclear lesion especially observed in HODGKIN's disease, consists in the appearance of one or more concentric bodies with a visible clear halo in the midst of the nucleoplasm (Fig. 1). The meaning of this alteration is not known; it has also been described in other human neoplasms by BERNHARD *et al.* (6–7).

In several cases, a number of different intranuclear inclusions were observed, similar to those described by BESSIS (9) in WALDENSTRÖM's syndrome. Apparently they are formed by an invagination of the cytoplasm into the nucleus. These inclusions, multiple and of large size, may occupy great part of the nucleoplasm (Fig. 2). The material included becomes unrecognizable, being transformed into a dense and homogeneous substance. These inclusions may later undergo a process of lysis, probably of enzymatic nature. In a case of lymphosarcoma, where the biggest vacuoles we have ever seen were observed in great number, the patient was followed up for 28 months with 19 aspiration biopsies. Despite the fact that the lesions were continuously encountered, there was an evident relation between the clinical condition of the patient and the size of the vacuoles. When the patient was in remission, it was difficult to find vacuoles, while in the event of a new relapse, the lesions became more evident in almost every one of the nuclei. It should be emphasized that no similar lesions were observed in the white blood cells of the patient.

*Nucleolar Lesions*

Occasionally the examination of the nucleolus may help to characterize malignant cells, by their enormous size, their deformities, and their plurality.

In a case of reticulosarcoma, the nucleoli, although not clearly visible with MAY-GRÜNWALD-GIEMSA stain, were quite evident, however, under Phase Contrast and more so under the electron microscope. Another very acute cas was characterized by a large number of nucleoli.

An alteration which sometimes has been observed in the neoplastic cell is a well-developed nucleolema (Fig. 3).

*Cytoplasmic Inclusions*

In a case of monocytoblastoma, well delimited fibrillar zones were observed in the cytoplasm of each cell. These consisted of parallel or ring shaped fibres (Fig. 4); vacuolar or lipid inclusions were




*Fig 1* Nucleus of STERNBERG cell, showing fibrillar body (arrow) within the nucleoplasm. × 22.000.

*Fig 2* Reticular cell with nucleus with large vacuole containing membranous debris and well delimited inclusions. × 10 000.

*Fig 3* Reticular cell showing dense nucleolonema. × 24.000.

*Fig 4* Part of fibrillar body within the cytoplasm of monoblast. 44.000




*Fig. 5.* Group of lymphoblasts showing large cytoplasmic inclusions made up of an amorphous material × 7.000.

*Fig. 6.* A big vacuole occupying the cytoplasm and pushing the nucleus (N) toward pole of the cell. 10.000.

*Fig. 7.* Tw "cup-shaped" mitochondrias. The enclosed cytoplasm shows glycogen granules. × 50 000.

*Fig. 8.* Cytoplasmic acuole containing esicules of homogeneous size and clear center 3..000

found amidst the fibrinoid masses, similar to those described by Bessis (8) in different human and murine leukemias.

In three cases of lymphosarcoma of different evolution large cytoplasmic inclusions were observed in nearly every cell. These occupied the main part of the cytoplasm, projecting the nucleus towards the periphery and displacing the cytoplasmic organelles. In two of the cases, these well-defined inclusions were formed by an amorphous material without any delimiting membrane (Fig 5) In the third case there was, however a well-delimited membrane (Fig 6)

The size, number and form of the mitochondrias can vary a great deal in the same lymph node and even in the same cell in general there are many large and swollen mitochondrias. In some cases, structural abnormalities are evident the cristae are found along the mitochondrial membrane or may acquire bizarre forms with glycogen accumulated within the ring (Fig 7)

*Cytoplasmic Vacuoles*

In two cases of reticulosarcoma and in two cases of lymphosarcoma, we have observed multiple vacuoles disseminated in the cytoplasm. These usually contain numerous vesicules of 400 A in diamenter having a double membrane and a clear center (Fig 8)

The origin of these formations is difficult to elucidate they may be multivesicular bodies or only the expression of a vacuolar degeneration The hypothesis that viral particles may be involved has already been discussed in a former paper This possibility can not be rejected a priori since in the patients it was possible to observe nuclear lesions similar to those brought about by known viruses nuclei with definite margination of the chromatin and clumps of granules in the midst of the nucleoplasm

We have described (27) a series of nuclear and cytoplasmic lesions, which were encountered in lymph nodes of different types of malignant human lymphomas. Although many of these lesions have already been described by others, in isolated cases or in general reviews, we are still in a stage of recopilation of data where every observation is still valuable and interesting In the first place attention must be drawn to the fact that these ultraestructural lesions were only observed in a small percentage of the specimens examined. These lesions were always present in one or more of the 54

cases of malignant lymphomas but were never seen in the 20 cases of benign hyperplasias studied.

In the second place, it must be stressed that we have observed lesions, which were often identical, in very different cases, as far as cytology clinical diagnosis and prognosis were concerned It is not easy to interpret these cellular lesions and their value. It is difficult to accept that they may be secondary to the treatment administered to the patients, since they have often been found previous to therapy Moreover during treatment, any change is in quantity and not in quality

Can these lesions be considered characteristic of neoplasia? It would seem difficult to admit that lesions so different one from the other may be primarily related to carcinogenesis. It would rather appear as if these were lesions secondary to a marked disorder in cellular metabolism and reflecting alterations at the molecular level.

As we have already pointed out, the identification of the etiological agent can be simple, when bacterias, fungi and parasites are involved, but can be very difficult in the case of viruses. However experiments carried out in mice in our laboratory by Drs. PASQUALINI *et al* (19) favor the viral etiology The inoculation of biopsy material into the spleen of BALB mice led to the development of leukemias of short latency (within 3 months) in 3 out of 31 cases, 2 hemocytoblastomas and 1 fibrosarcoma. Non malignant human control tissue gave negative results.

It must be stressed that in none of the cases studied, it has been possible to observe typical viruses. The presence of vesicules more or less similar to some viruses and identical to those observed by SORENSEN (24) in human myelomas is most interesting but needs further biological confirmation. Some of the nuclear and nucleolar lesions described (concentric bodies, margination of the chromatin, cluster of granules) point to the existence of processes similar to those observed in murine leukemias and experimental viral infections. However these are only morphological analogies which do not permit more than a working hypothesis.

*Liberation Phase*

This phase is the one responsible for the passage of lymphoid elements from the lymph node to the peripheral blood. Very little is known about the laws which govern lymphocytic function. There

are enormous lymphoid hyperplasias, in which the elements do not pass to the peripheral blood and again, at other times, there may be a very sudden liberation of immature and mature cells into the blood.

In other cases the cells are liberated as soon as they are formed This is what happens in infection mononucleosis and lymphocytosis.

*Cytogenetic Study of Lymph Nodes*

As ASTALDI (3) pointed out in the opening remarks of the Symposium on Chromosomes, in the IXth Congress of the European Society of Hematology in 1963 interest in this branch of hematology is increasing every day

In collaboration with Dra SALUM (23) we have determined the karyotype in benign and malignant lymphoid hyperplasias, trying to relate karyotype to evolution of the disease. In 15 cases of lymphopathies of different nature, we have obtained preliminary data which allows us to presume that there is a cellular imbalance accompanying abnormal metamorphosis, as a common feature. Each case has its own peculiar cytogenetic profile and no definite alteration has been observed among the karyotype of different lymph nodes of histologically similar processes The presence of heteroploidy does not necessarily mean that the process is malignant and is not related to clinical evolution. It would seem to be associated with the acute phase of the cellular proliferation.

The morphological abnormalities of the chromosomes were striking in the following cases

A chronic lymphoid leukemia in which a tiny acrocentric chromosome of the 21–22 group was observed in 34% of the metaphases, relating it to the CH chromosome described by GUNZ *et al* (15) In a case epidermoid carcinoma chromosomes abnormal in their morphology and a great number of fragments were observed. A case of Leukosarcomatosis previously treated with cobalt-therapy presented contracted and broken chromosomes.

*A high % of endomitosis was found in cases previously treated* with Roentgen, $P_{32}$, and antimitotic drugs.

In the determination of mitotic indexes, a correlation of high indexes in tissue cultures and in the acute period of ganglionar grouth has been found although no parallelism between malignancy according to the clinical evolution, and high mitotic indexes were observed.

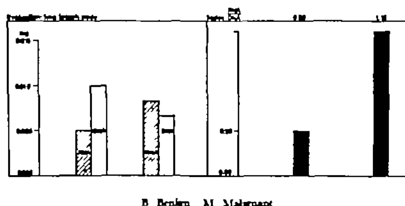
### *Nucleic Acids and Malignant and Benign Hyperplasias of Human Lymph Nodes*

Another investigation which helps to characterize certain cancerous tissue is the determination of the quantitative relation between RNA and DNA.

In collaboration with Drs. SRULIJES (25–26) nucleic acids have been isolated from different cases of lymphoid hyperplasias, determining the $\frac{RNA}{DNA}$ indexes by chemical methods, and establishing a comparison between malignant and benign hyperplasias (see Table I). In the benign hyperplasias the quantity of DNA is twice the quantity of RNA, while in the malignant hyperplasia the quantity of RNA is superior to the amount of DNA therefore the $\frac{RNA}{DNA}$ index is greater in the cases of malignant hyperplasias than in the cases of benign hyperplasias.

*Table I*

Interrelationship between RNA and DNA in lymph node hyperplasias.



B Benign M Malignant

This study was statistically evaluated (see Table II) showing that the superior limit of the index $\frac{RNA}{DNA} = 0{,}90$ is not surpassed by any of the 10 benign cases and it is exceeded in 5 of the 23 malignant cases this difference is very significant

$$t = 3\ 70 \quad p < 0\ 001$$

*Malignant Hyperplasias which Exceed the Superior Limit of the Index*

$$\frac{RNA}{DNA} = 0\ 90\ (t = 3\ 70\quad p < 0.001)$$

*Table II*

| Number of cases | Diagnosis | $\frac{RNA}{DNA}$ Index |
|---|---|---|
| 1 | *Reticulolymphosarcoma* | *1 16* |
| 1 | Hemocytoblastoma | 0.92 |
| 1 | Metastasis of epithelial tumor | 1 16 |
| 1 | Acut lymphoid leukemia | 2.33 (W B.C.) |
| Total | | range |
| 10 | Benign hyperplasias | 0.24–0.77 |
| 23 | *Malignant hyperplasias* | 0.35–2.33 |

*Ganglionar Cytoimmunology*

At present, great importance has been assignated to the lym phatic system in immunological processes, as it has been pointed out by Dameshek (13) Actually the presence of a swollen lymph node is considered as a defensive reaction, possibly conditioned by an immunological mechanism These reactions, sometimes fugaci ous, can nevertheless provoke hyperplasias of great mitotic activity even leading to temporary diagnostical doubt. The identification of the agent acting as antigenic stimulus confirms the diagnosis at times it is easily discovered or only after very patient investiga tion These adenopathies may persist during long periods of time conserving the same cytological characteristics. Logically the dis-covery of the etiological cause is decisive for the future evolution.

It is not always possible to demonstrate a definite immuno-logical origin, but new contributions in immunological techniques have again raised hopes of decisive advances.

With the collaboration of Dr Bachmann (4–5) we have not been able, up to now to demonstrate an immunological etiology with the techniques used to detect specific antibodies, either for lack of those antibodies, or else because of a phenomenon of de layed immunity

As Burnett reports (11) in certain reactions of the lymphatic system the appearance of cellular clones would be at the basis of the immunological mechanism. The stimulation of the lymphatic sys-

tem by means of different antigens (foreign proteins, bacterial anti gens, skin grafts) brings about images of hyperplasias, in which embryonal cells may be observed. The denomination of these cells has aroused a terminological controversy (FAGREUS's hemocytoblasts [14] DAMESHEK's immunoblasts [12] POLICARD s hyperbasophylic cells [22] AMANO s lymphogonias [1] LENNERT's germinal cells [16]) Simple reactional hyperplasias experimentally transformed by means of a prolonged antigenic stimulus in to blastomatous processes have been reported (METCALF 18 DAMESHEK, 12)

Finally it must not be forgotten that the stimulation of the lymphatic system in the auto-immune mechanism, conditions hyperplasias in certain lymphoid focuses scattered in different parts of the organism, MACKAY (17) For these reactions to occur the antigen, the site of formation of the antibodies, the cellular changes, mutations, and the formation of special clones, must be taken into account. Cytological studies lead to the determination of the participation of the lymphoid tissue in these reactions. With the immunofluorescent techniques, the antibody forming cells can be localized. On the other hand, repeated controls of the cytological images by means of serial punctures, show simple generalized hyperplasias that can regress when the antigenic focus is eliminated (in our experience teeth, sinusitis, gall bladder empyema) while in other occasions these images persist, the antigenic focus having not been discovered leading finally to a malignant transformation, clinical as well as cytological Three different examples will illustrate these points (see Table III) the first is a process which had overtaken great part of the lymphatic system, with enormous lymph nodes in the neck region and a tumoral like mass in abdomen. The histology and cytology showed a benign reactional hyperplasia mainly of lymphocytes and prolymphocytes. When the very extensive dental septic focuses were eliminated, the tumoral mass and lymph nodes disappeared completely

In the second case, the initial histological and cytological picture led us to suspect a benign evolution it was not possible to individualize septic or antigenic focuses. In spite of this, there was a final malignant evolution, with generalization of the process, bone invasion and malignant transformation of the lymphoid elements.

The third case which was characterized by enormous lymph nodes in neck and hyperplastic tonsils, the aspiration biopsy revealed a malignant lymphoma, with the presence of embryonal

## *Summary*

The author presents his contribution to the diagnosis of lymphopathies by means of the lymph node puncture which he initiated in 1934 The possibilities of more ample cytological examination by means of new techniques and scientific discipline are discussed, referring especially to the contribution of cytochemistry biochemistry RNA and DNA dosification, karyotype determination, cell cultures, immunology enzymology and electron microscopy

The pplication of these different methods for the study of the lymphopathies is discussed with special emphasis on the contribution of the electron microscope for the diagnosis and prognosis of these diseases.

## *Zusammenfassung*

Der Autor orientiert über seinen Beitrag zur Diagnose der Lymphopathien mit *Hilfe der von ihm 1934 eingeführten Lymphknotenpunktion. Die Möglichkeiten einer* umfassenderen zytologischen Beurteilung mit neuen Methoden werden erörtert, wobei besonders hingewiesen wird auf Zytochemie, Biochemie Bestimmung von RNA und DNA, Feststellung des Karyotypes, Zellkulturen, Immunologie Enzymologie und Elektronenmikroskopie Die Anwendung dieser erschiedenen Methoden zur Untersuchung der Lymphopathien wird diskutiert, wobei der Beitrag der Elektronenmikroskopie zur Diagnose und Prognosestellung dieser Krankheiten hervorgehoben wird.

## *Résumé*

L auteur présente sa contribution au diagnostique des lymphopathie à l'aide de la ponction des ganglions lymphatiques qu il a introduite en 1934 Les possibilités d'un examen cytologique plus ample à l'aide de nouvelles tecniques et de nouv Iles disciplines scientifiques sont discutées, particulièrement les contributions de la cytochimie, de la biochimie de la dosification de l'ARN et de l'ADN de la détermination du karyotype des cultures cellulaires, de l'immunologie, de l'enzymologie et des examens au microscope électronique. L application de ces différentes méthodes à l'étude des lymphopathies est discutée et la contribution du microscope électronique au diagnostique et au prognostique de ces maladies est spécialement soulignée.

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# Cell Proliferation in Leukemia During Relapse and Remission

## I DNA and RNA Synthesis of Leukemic Cells in the Bone Marrow *in vitro*

J R. Schmid J M Kiely W N Tauxe and C.A. Owen Jr

In 1846, Virchow (1) described leukemia as a disturbance of the normal steady-state condition of white blood cells, initiated by uncontrolled and accelerated cellular proliferation. Today considerable evidence exists that leukemic cells are not proliferating more actively than the corresponding normal cells (2–5) A study of changes in the proliferation of leukemic cells at the time of early remission or just prior to relapse may give information about the mechanism of the disease process itself. This was attempted, in the work reported herein, by studying nucleic acid synthesis in short term *in vitro* incubations of bone marrow from patients with different types of leukemia, with DNA and RNA precursors.

### *Patients and Methods*

*Patients.* This study included 20 patients with different types of leukemia and one patient in 'preleukemic' state (four sternal marrow aspirates were obtained from one patient and one aspirate was obtained from each of 20 patients). *In vitro* DNA synthesis was investigated, by using thymidine-$^3$H, in all the specimens *in vitro* RNA synthesis could be evaluated, by using uridine-$^3$H in only 17 instances, for technical reasons discussed below

Acute types of leukemia (myeloblastic, monoblastic, or lymphoblastic) were present in 14 patients, and the leukemic process was chronic (myelocytic or lymphocytic) in 6 patients. In one patient, preleukemic state existed with refractory anemia and "left-shifted" marrow containing increased numbers of immature cells acute stem cell leukemia developed in this patient 14 months after the marrow study reported here.

*Radioactive reagents.* Thymidine-$^3$H and uridine-$^3$H, both with specific activity of 1.9 cu/mmole and concentration of 1 mc/ml, were obtained from Schwarz BioResearch, Inc. (Orangeburg, New York)

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*Method of preparing autoradiographs.* A 0.5- to 1.0-ml sample of marrow was obtained by sternal aspiration, with heparin as anticoagulant. Exact volumes of this sample were pipetted into siliconized incubation containers to which the appropriate tritiated nucleoside was added so that the final concentration was 0.8 $\mu$c/ml. The marrow sample was incubated at 37 °C with gentle shaking *for exactly 1 hour and then was smeared on* gelatin-coated slides, air dried, fixed in methanol, and mounted (in the dark) with Eastman-Kodak AR 10 stripping film as described by Pelc (6). The mounted slides were air dried, alcohol dried, and stored in refrigerator at 4 °C. After the appropriate period of exposure, the films were developed at 18 °C with Kodak D-19 developer and Kodak acid fixer. For staining with Giemsa stain, the slides were placed in a solution, at 18 °C, buffered t pH 6.5

The evaluation of the slides was based on the percentage of labeled cells of each type. A cell was defined as labeled only if the number of overlying grains was t least twice the average background noted in noncellular zones.

*Method of expressing results.* A 1-hour DNA synthesis index was expressed as the percentage of blast cells labeled. A 1-hour RNA synthesis rate was determined by plotting (on logit ruling) the percentage of labeled blast cells as function of the exposure time of the film. T compare results in leukemic cells obtained from different patients, the rate of RNA synthesis in each marrow sample was computed and evaluated separately for blast cells and for myelocytes in the following manner. First, the RNA synthesis rates were determined for the abnormal cells and for normal myelocytes in the same marrow smear. Second, the average value obtained for the myelocytes in the abnormal marrow was compared with the average value determined for myelocytes in normal marrows when the same incubation period, initial concentration of the precursor and exposure period of the film were used. A correction factor was computed for the abnormal marrow based on the ratio of percentage of labeled myelocytes in the normal to that in the abnormal marrow. Third, the measured value for the abnormal cell was multiplied by this correction factor. Thus, the corrected value expressed the rate of RNA synthesis by the abnormal cell in relationship to an average value in normal marrows (unpublished data).

## *Results*

*One-hour DNA synthesis index.* The mean percentage of blast cells labeled by tritiated thymidine from patients with acute lymphoblastic and monoblastic leukemia was almost double that of blast cells from patients with acute myeloblastic leukemia (10.5% versus 5.7%). The percentages of labeled blast cells from three patients with chronic myelocytic leukemia (two of them developing a terminal blastic transition) were also approximately twice as high as those noted in acute myeloblastic leukemia. The lowest values of the entire series, 0.6 to 1.5% labeling of blast cells, were found in marrows from the three patients with chronic lymphocytic leukemia. The range and mean values for the different leukemic groups are summarized in the table. In one patient entering the blastic transition of chronic myelocytic leukemia, the percentage of labeling with thymidine-$^{3}$H was determined simultaneously in blast

*Table*

Range and mean values of the DNA synthesis index (thymidine-$^{3}$H labeling) for the different leukemia groups studied.

| Type of leukemia | No. of studies | Labeled blast cells in percent range | mean |
|---|---|---|---|
| Acute myeloblastic | 3 | 5.0– 6.5 | 5.7 |
| Acute lymphoblastic and monocytic | 14 | 6.5–18 | 10.5 |
| Chronic myelocytic | 1 | | 14 |
| Chronic myelocytic entering blastic phase | 2 | 10.5–12.5 | 11.5 |
| Chronic lymphocytic | 3 | 0.6– 1.5 | 0.9 |
| Preleukemic | 1 | | 16 |

cells of the bone marrow and of the peripheral blood a markedly lower value was noted in the latter (12.5 / versus 2.0 /)

In marrow from 13 patients who had acute leukemia and who were taking chemotherapeutic agents at the time of the marrow aspiration, a mean labeling of 10.9 / was found in contrast to a mean of 5 6 / in marrow from two untreated patients.

The marrow from the 5 patients who were entering or had entered a remission phase within 1 month after this study showed a mean labeling of 14.9 / of blast cells (range, 12 to 18) compared to a mean of 7.5 / (range, 4.8 to 13) for 10 samples taken during relapse and 8.0 / for one sample taken prior to relapse.

*Correlation with clinical studies* Two cases, both of acute leukemia, will be considered by way of example. In one, the patient was studied just prior to remission in the other the patient had progressive and rapidly fatal disease.

A 44-year-old man gave history of anemia, weight loss, and fatigue during the 3 months prior to admission. He had been treated, by his local physician, with 6-mercaptopurine for 1 month. Laboratory findings included hemoglobin concentration, 9.8 g/100 ml of blood platelet count, 299,000 mm$^{3}$ leukocyte count, 3600 mm$^{3}$ with 43% lymphocytes, 11.5% monocytes, 43.5% neutrophils, 1% basophils, 0.5 early myelocytes, and 0.5% late myelocytes. Blast cells were still abundant in the bone marrow constituting 30% of all nucleated elements. Thymidine-$^{3}$H label was taken up by 18% of these blast cells. The patient went into remission 1 month later and remained well for 7 months on maintenance regimen of 6-mercaptopurine.

A 63-year-old woman gave history of ecchymoses of 1 week duration. Previously she had been in excellent health but had noted increasing fatigue during the past few months. Laboratory examinations revealed hemoglobin concentration, 9.4 g/100 ml of blood, platelet count, 39 000 mm$^{3}$ leukocyte count, 31 300 mm$^{3}$ with 13.5% lymphocytes, 0.5% neutrophils, 76.0% blast cells, 10% prolymphocytes, and 1 normoblast per 200 leukocytes. The bone marrow was hypocellular and consisted almost exclusively of large blast cells, of which only 4.8% became labeled following the 1-hour incubation with thymidine-$^{3}$H. Treatment with 6-mercaptopurine and prednisone was ineffective and the patient died after 20 days.

*One-hour RNA synthesis rate* The incorporation of uridine-³H by blast cells was compared to that by myelocytes. Therefore, data could be obtained only in those samples of marrow in which a sufficient number of myelocytes were present.

In all patients with acute leukemia, a greater percentage of myelocytes than blast cells were labeled with uridine-³H. Leukemic blast cells incorporated this nucleoside at a rapid rate, similar to that of normal immature cells from control subjects (Figure). This finding was unexpected because the values for DNA synthesis in blast cells from patients with acute leukemia were found to be uniformly low compared with immature cells of normal marrow.

A different pattern of labeling with uridine-³H was found for blast cells in patients with chronic lymphocytic leukemia. In the three patients investigated the incorporation of the RNA precursor was consistently lower than that noted in patients with acute leukemia (Figure).

## *Discussion and Conclusions*

*In vitro DNA synthesis* Similar indices of DNA synthesis by blast cells were found in acute leukemias of different morphologic types. Differences were noted in the two types of chronic leukemias. The DNA indices of blast cells from 3 patients with chronic myelocytic leukemia were higher and the values in 3 patients with chronic lymphocytic leukemia were lower than those in the acute leukemia group. In all of the leukemia patients, the DNA indices were lower than those in normal immature cells. This is in keeping with the current concept that leukemic cells grow slowly and have a long generation time (2, 7 8).

Higher DNA indices were noted in 13 patients receiving antileukemic therapy indicative of a higher proliferative potential of the leukemic cells, compared with two patients not treated prior to the study. This finding is somewhat surprising since antileukemic agents have been assumed to exert their effect by inhibiting cellular proliferation.

Results of this study are particularly interesting in regard to changes in the proliferative potential of leukemic cells at the beginning of remission. Ten patients with progressive and rapidly fatal disease were found to have low DNA indices of the marrow blast cells (mean, 7.5 %) while 5 patients in early remission gave

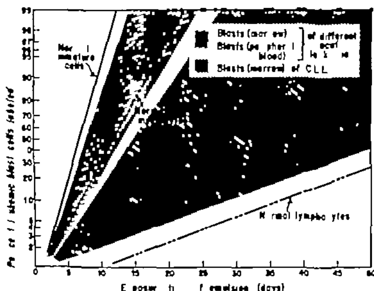



*Figure.* Percentage of cells labeled with uridine-³H (on logit ruling) as function of the exposure time of the film. Blast cells of patients with acute leukemia label heavily with this RNA precursor and only blast cells of chronic lymphocytic leukemia (CLL) give a separate pattern of labeling (open triangles).

higher values (mean, 14.9 %). The latter value was obtained at a time when the bone marrow was still heavily infiltrated with leukemic blast cells. This observation is also at variance with the concept of remission in leukemia being the result of inhibition of rapidly proliferating leukemic cells and relapse being due to the return of rapidly dividing blast cells.

Leukemic cells gradually disappear from the marrow at the time of remission and become replaced by normal elements (9). Studies of the DNA synthesis period (4, 5, 10) and of the mitotic index (11) have established that the proliferative potential is considerably lower in leukemic cells of any type than it is in normal immature marrow cells. It is probable that leukemic cells accumulate in the marrow in spite of their relatively low proliferative potential because their maturation and subsequent release is impaired.

*Chemotherapy.* At the present time, knowledge about the mechanism of action of antileukemic agents is still incomplete. Inhibition of cell proliferation alone does not completely explain remission of the leukemic process, since normal marrow cells, which have a higher proliferative potential, predominantly would be inhibited.

It has been assumed that antileukemic agents are primarily effective by inhibiting DNA replication prior to cell mitosis. This mechanism might explain our observation that leukemic patients with a high DNA synthesis index seem to be more likely to have a remission than do those with a low index. However some specific effect on the leukemic cell would still have to be implicated, since normal cells with high DNA indices may increase in the marrow at the time of remission while the patient continues to take the chemotherapeutic agent.

The mode of action of antileukemic agents is probably complex and varies from one drug to another (12, 13) but they all influence nucleic acid metabolism. It has been found, for example that actinomycin D inhibits primarily RNA synthesis in certain cell systems (12) or specifically the DNA-dependent RNA synthesis (14–16) and DNA replication itself only secondarily Other drugs, like 6-mercaptopurine or cyclophosphamide, affect mainly the metabolism of DNA itself (17 18) the exact mechanism being unknown. Conversely hydrocortisone was found to induce an increase in DNA synthesis and mitotic index (19)

*In vitro RNA synthesis* In contrast to the low DNA synthesis indices noted, our results with uridine-$^3$H indicate that a high rate or RNA synthesis is a feature of leukemic cells (Figure) In normal cells the rates of DNA and RNA synthesis are both high in immature forms and decrease as cell maturation proceeds, suggesting that, in normal marrow cells capable of undergoing mitosis, RNA synthesis is primarily related to the process of cell proliferation. In leukemic blast cells we found a dichotomy in the synthesis of the two nucleic acid fractions.

One conclusion suggested by these findings is that, in the induction of remission of leukemia, inhibition of some step of RNA synthesis may be more important than inhibition of cellular proliferation by direct interference with DNA synthesis. Indeed, the ratio of RNA synthesis to DNA synthesis was greater in the group of acute leukemias in relapse than in those in early remission. The higher DNA synthesis indices of blast cells found in marrows of patients entering remission seem indicative of a more normal pattern of DNA and RNA synthesis. As an early response to successful antileukemic therapy a different population of immature cells, characterized by a high proliferative potential and the ability to differentiate, may appear in the marrow

*An analogy to viral leukemogenesis* The high rate of RNA synthesis found in leukemic cells cannot be accounted for by either of the two factors responsible for this finding in normal marrow cells: a high proliferative potential and production of proteins. It is conceivable that a third factor may explain the high RNA turnover of these leukemic cells. According to present concepts, a viral factor may be involved in human leukemogenesis (20, 21), perhaps after enhancement by cocarcinogenic factors (22–30). It is known that malignant transformation in cells in tissue culture may occur following infection with certain viruses whose nucleic acid may become partially integrated into the cell genome (20, 31–35). In experimental studies with tissue cultures, one of the first changes observed following viral infection was the appearance of clones of transformed cells able to proliferate indefinitely (33, 36), probably due to a loss of regulatory systems (20, 37, 38). Inability to differentiate was usually a concomitant feature as was a prolonged generation time (35, 39). To a certain extent, these findings are comparable with the data obtained in the present study of human leukemia. The higher proliferative potential of leukemic cells prior to remission may indicate a change toward a more normal pattern.


### *Summary*

Samples of bone marrow from 20 patients with different types of leukemia were studied by autoradiography after *in vitro* incubation with tritiated thymidine and uridine. Thymidine uptake by leukemic blast cells was considerably less than that by normal blast cells, whereas uridine incorporation was greater in leukemic blast cells. The average DNA synthesis index of blast cells from patients with acute leukemia entering remission was almost double that of blast cells from patients with progressive disease. The data suggest that inhibition of cell proliferation by antileukemic agents may not be the major basis of remission in acute leukemia.



### *Zusammenfassung*

Knochenmark von 20 Patienten mit verschiedenen Leukämieformen wurde nach Inkubation *in vitro* mit Tritium-markiertem Thymidin und Uridin autoradiographisch untersucht. In Paraleukoblasten war die Thymidinaufnahme beträchtlich niedriger als in normalen Leukozytenvorstufen, während der Einbau von Uridin in Paraleukoblasten größer war. Der mittlere DNA-Synthese-Index der Paraleukoblasten von Patienten mit akuter Leukämie in beginnender Remission war nahezu doppelt so groß als bei Patienten mit fortschreitender Erkrankung. Die Ergebnisse lassen vermuten, daß eine Hemmung der Zellproliferation durch antileukämische Medikamente der Remission einer akuten Leukämie nicht in erster Linie zugrunde liegt.

## *Résumé*

La moelle osseuse de 20 malades atteints de différentes espèces de leucémie a été examinée à l'autoradiographie après une incubation *in vitro* à l'aide de thymidine tritiée. La résorption de thymidine était beaucoup moins forte dans les paraleucoblastes que dans les précurseurs des leucocytes normaux, l'incorporation d'uridine par contre était plus forte dans les paraleucoblastes. *L'index moyen de la synthèse de l'ADN* des paraleucoblastes de malades atteints de leucémie aiguë début d une rémission était à peu près deux fois plus grand que celui de malades dans un état avancé de la maladie. Ces résultats semblent indiquer que l'inhibition de la prolifération des cellules par des médicaments anti-leucémiques n'est pas, en premier lieu, à la base d'une rémission dans les leucémies aiguës.


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# Aggregation und Desaggregation von Erythrozyten

A. M. Ehrly und H. E. Müller

Für die erstmals von Cocchius (1) beschriebene »körnige Strömung« des menschlichen Blutes ist später von Knisely (2) der Ausdruck »sludged blood« geprägt worden. Darunter wurde die Zusammenballung von Erythrozyten (Aggregation) verstanden, die *intra vitam* mittels der kapillarmikroskopischen Betrachtung kleinster Gefäße der Conjunctiva bulbi, des Nagelfalzes und der Haut bei einer Reihe von Erkrankungen (Schock, Verbrennung, Thrombose, Herzinfarkt, Plasmozytom u. a.) gefunden wurde. Während von den meisten Autoren (z. B. 3, 2, 4) angenommen wird, daß aggregierte Erythrozyten im kapillaren Bereich zu Störungen der Mikrozirkulation führen können, herrscht über die Art der Erythrozytenaggregate noch keine Übereinstimmung. Thorsen und Hint (5) sowie Bergentz und Fahraeus (6), Ditzel (7) und Hirschboeck und Woo (8) konnten bei ihren Untersuchungen nur geldrollenartige Aggregate finden; andere (9, 10) dachten an das Vorliegen verschiedener Aggregatformen, da sie vereinzelt eine fehlende Korrelation zwischen klinisch beobachtetem blood-sludge und der Blutkörperchensenkungsgeschwindigkeit (BSG) sahen. In Fortführung eigener Untersuchungen soll in der vorliegenden Arbeit durch vergleichende Viskositäts- und BSG-Versuche sowie durch Modellversuche die Frage nach verschiedenen Erythrozytenaggregaten sowie deren Desaggregierung näher untersucht werden. Die Erythrozytenaggregate wurden *in vitro* durch eine besondere Ausstrichtechnik sichtbar gemacht.

## *Methodik*

Es wurde Normalblut und Blut von Patienten mit beschleunigter BSG und kapillarmikroskopisch sichtbarem Sludge (z. B. bei akuter Bronchopneumonie, β-Plasmozytom) untersucht. Die Proben wurden mit Na-Oxalat ungerinnbar gemacht. In einem

Teil der Fälle wurde die Aggregation von Erythrozyten in vitro durch Zusatz von Human-Fibrinogen (Behringwerke Marburg) oder von hochmolekularem Dextran mit einem mittleren Molekulargewicht von 71000 (Firma Knoll AG, Ludwigshafen/Rh.) in Konzentrationen von 500 bis 1000 mg% hervorgerufen. Die Viskositätsmessungen wurden bei 37 °C ± 0,1 °C im modifizierten (11) Ostwald-Kapillarviskosimeter mit dem Kapillarradius 0,025 cm durchgeführt (Doppelbestimmungen). Die Durchflußzeiten lagen zwischen 60 und 180 s. Die Blutproben wurden auf einen Hämatokrit (HT) von 40% eingestellt; die Messung des Hämatokrits (HT) erfolgte nach Wintrobe. Das Verhältnis der Durchlaufzeiten von Blut zu destilliertem Wasser unter gleichen Bedingungen wurde als relative Viskosität angegeben. Die BSG wurde mit Westergren-Röhren bestimmt.

Natrium-oleat (rein, Firma Riedel de Haen, Seelze) wurde den Blutproben in Konzentrationen bis 90 mg% zugefügt (12). Vergleichsweise wurden in parallel laufenden Versuchen Dodekansulfonat (Firma Henkel, Düsseldorf), Cetyltrimethylammoniumbromid (Firma Schuchardt, München), Na-aescinat (Reparil®, Firma Madaus, Köln) und Na-desoxycholat (Firma E. Merck, Darmstadt) in äquimolaren Mengen verwendet. Dextrose wurde in Konzentrationen von 0 bis 6,4 g% zum Normalblut zugegeben. Erythrozytensediment wurde nach Abhebung des Plasma mit NaCl-Lösungen fallender Konzentrationen (0,85 bis 0,45%) vermischt.

Die «Blutausstriche» wurden wie folgt hergestellt: Auf Objektträgern, die vorher mit Äther entfettet wurden, wurde an einem Ende ein Tropfen der Blutprobe aufgebracht und durch einen konstanten schwachen Luftstrahl so über den Objektträger verteilt, daß ein dünner Blutfilm entsteht. Diese «Ausstriche» müssen sofort unter einem Phasenkontrastmikroskop auf das Vorhandensein von Erythrozytenaggregaten untersucht werden, bevor der dünne Blutfilm eintrocknet. Geschieht dies nicht, dann kann die Wasserverdunstung eine Konzentration der Erythrozyten bewirken und dadurch eine Aggregation vorgetäuscht werden. Bei vollständig eingetrockneten Ausstrichen treten eine Reihe von Kunstprodukten (u.a. Brückenbildungen zwischen den einzelnen Erythrozyten) auf.

Abgesehen von den Viskositätsmessungen erfolgten alle Untersuchungen bei Raumtemperatur (21 °C). Die kapillarmikroskopischen Untersuchungen wurden mit einem Stereomikroskop (Firma Zeiß, Oberkochen) bei einer Vergrößerung von 80 × durchgeführt.

## *Ergebnisse*

### *a) Phasenkontrastmikroskopische Untersuchung der Blutausstriche*

Vom Blute von Patienten, die Zeichen einer vermehrten Erythrozytenaggregationen aufwiesen (hohe BSG, kapillarmikroskopischer blood-sludge) wurden Blutausstriche angefertigt. Eine Aggregation von Erythrozyten konnte auch im Blut gesunder Probanden ohne BSG-Beschleunigung und ohne klinischen blood sludge durch Zugabe von Humanfibrinogen oder hochviskösem Dextran in Konzentrationen bis 1000 mg% provoziert werden. Eine bereits vorhandene Aggregation wird durch die Zugabe dieser Substanzen noch verstärkt. Die zu beobachtenden Aggregate sind in Abb. 1 schematisch aufgezeichnet. Blut der Patienten mit klinisch deutlichem blood sludge wies Aggregate in Form von Geldrollen auf (primäre Geldrollenaggregate). Die Zahl der Geldrollen im Ver

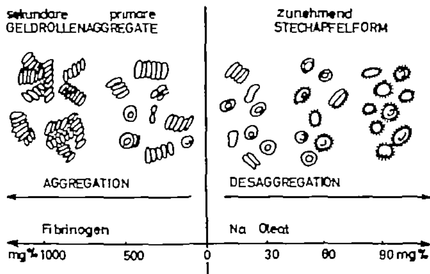



Abb. 1 Schema der mikroskopisch sichtbaren Aggregation von Erythrozyten nach Fibrinogenzusatz und der Desaggregation durch steigende Konzentrationen von Na-oleat.

hältnis zu den frei liegenden Erythrozyten ist um so größer, je ausgeprägter die Senkungsbeschleunigung und der sludged blood-Effekt ist, bzw. je höher die Konzentration an Fibrinogen oder Dextran ist. Die einzelnen Erythrozyten in den Geldrollen (rouleaux) liegen bei steigenden Konzentrationen dieser hochmolekularen Stoffe dichter aneinander und die Geldrollen ihrerseits bilden dann durch Zusammenlegung zu unregelmäßigen Haufen größere Aggregate (sekundäre Geldrollenaggregate) (s. Abb. 1). Diese sekundären Geldrollenaggregate ließen sich neben primären Aggregaten bei stark ausgeprägtem Sludging nachweisen.

Zugabe von steigenden Mengen Na-Oleat, Dodekansulfonat oder Cetyltrimethylammoniumbromid führen zu einer Desaggregation bereits vorhandener primärer oder sekundärer Geldrollenaggregate (Abb. 1 und 2). Dabei werden die Geldrollenketten kürzer und die Zahl der Einzelerythrozyten nimmt gegenüber der Zahl der Geldrollen wieder zu. Es kommt bei stärkeren Oleatkonzentrationen zu zunehmender Stechapfelbildung. Vereinzelt erscheinen die Erythrozyten der noch teilweise vorhandenen kurzen Geldrollenketten ebenfalls stechapfelförmig. Bei hohen, jedoch nicht lytischen Konzentrationen (12, 13) von Oleat (100 mg%) oder

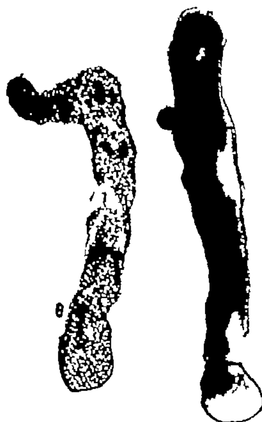


Abb. 2 Makroskopische Aufnahmen von Blutausstrichen. Links stark aggregiertes Blut. Rechts Blut des gleichen Patienten nach Zugabe von Na-oleat (vollständige Desaggregation)

anderen oberflächenaktiven Substanzen sind alle Erythrozyten stechapfelförmig. Sie lassen mikroskopisch keinerlei Aggregation erkennen.

*b) Veränderungen der Viskosität, der BSG und des HT nach Zugabe von Na-Oleat.* In Abb. 3 sind die Veränderungen der Viskosität, der BSG und des HT aufgezeichnet, wie sie nach Zusatz steigender Mengen von Oleat zum Blut eines Patienten mit BSG-Beschleunigung und kapillarmikroskopisch nachweisbarem blood-sludge eintreten. Steigende Konzentrationen von UFS führen zunächst zu einer Minderung der Viskosität des Gesamtblutes, bei höheren Dosen von Oleat steigt die relative Viskosität jedoch deutlich an. Die BSG wird durch steigende Oleatkonzentrationen deutlich gehemmt (14), bei einer Konzentration über 60 mg ist die Blutsenkung

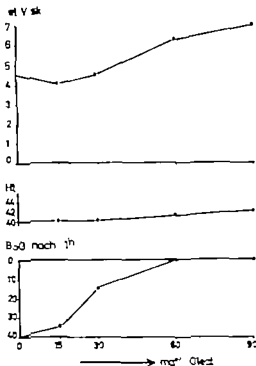



Abb 3. Verhalten der relativen Viskosität, des Hämatokrits und der Blutsenkungsgeschwindigkeit eines an Erythrozytenaggregaten reichen Blutes nach Zusatz steigender Konzentrationen von Na-oleat.

völlig aufgehoben. Der Hämatokrit steigt bei höheren Dosen geringgradig an. Qualitativ ähnlich wirken Dodekansulfonat und Cetyltrimethylammoniumbromid. Im Gegensatz dazu zeigen andere oberflächenaktive Stoffe, wie Na-desoxycholat und Na aescinat keine sichere desaggregierend oder senkungshemmende Wirkung

*c) Verhalten der Erythrozytenaggregate der Viskosität der BSG und des HT nach Zusatz hypertoner Lösungen.* Eine Desaggregation von Geldrollenaggregaten unter Bildung von stechapfelförmigen Erythrozyten läßt sich auch durch hypertone Lösungen, z. B. Glukose, erreichen. Abb 4 zeigt den Einfluß von Dextrose in Konzentrationen bis 6,4 g% zum Blute einer gesunden Person auf Viskosität, BSG und HT Während die Viskosität, besonders bei hohen Konzentrationen deutlich ansteigt und die Blutsenkung etwas gehemmt wird, nimmt der HT deutlich ab Mikroskopisch erkennt man im Blut dieser Probanden teilweise primäre Geldrollenaggregate die nach

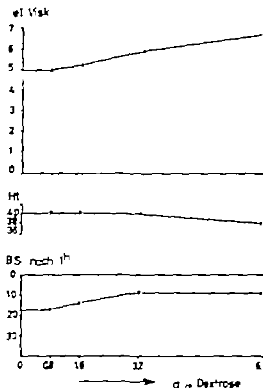



*Abb 4* Verhalten der relativen Viskosität, des Hämatokrits und der Blutsenkungsgeschwindigkeit bei steigenden Konzentrationen von Glukose.

Zusatz von hypertoner Dextroselösung verschwinden. Dafür nimmt die Zahl der stechapfelförmigen Erythrozyten zu.

*d) Der Einfluß hypotoner NaCl Lösungen auf die Viskosität den HT und auf Erythrozytenaggregate* Werden zu Blutproben mit deutlicher Erythrozytenaggregation und klinisch ausgeprägtem blood-sludge nach dem Zentrifugieren an Stelle des abgeheberten Plasmas NaCl Lösungen in fallenden Konzentrationen (von 0 85 bis 0,45 %) zugegeben, dann bewirkt dies zunächst eine deutliche Verminderung der ursprünglichen Viskosität bei geringgradig hypotonen Lösungen (Abb. 5) Verringert man die NaCl-Konzentrationen in den zugesetzten Lösungen noch mehr dann steigt die Viskosität wieder an bis letztlich die lytische Grenzkonzentration erreicht wird. Der HT nimmt deutlich zu. Mikroskopisch ist eine Desaggregation vorhandener primärer und sekundärer Geldrollenaggregate und ein Auftreten von Sphärozyten nach Zusatz hypotoner Kochsalzlösung zu erkennen.

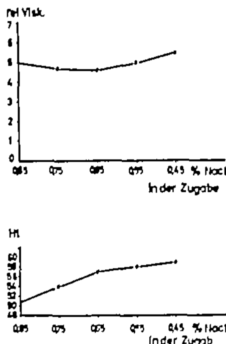



*Abb. 5.* Verhalten der relativen Viskosität und des Hämatokrit eines an Erythrozytenaggregaten reichen Blutes nach Zugabe von hypotonen NaCl-Lösungen an Stelle des Blutplasmas.

## *Diskussion*

Es konnte gezeigt werden, daß *in vitro* durch Zugabe von Fibrinogen oder hochmolekularem Dextran Erythrozytenaggregate auch im Blute gesunder Probanden entstehen können und daß der Grad der Aggregation im Blut von Patienten mit z. B. Pneumonien, Thrombosen, Plasmozytom u. a. durch Zugabe der oben genannten Substanzen verstärkt werden kann. Dabei kommt es, wie schon Thorsen und Hint (5) zeigen konnten, zu einer stetigen Zunahme der Aggregate in Form von Geldrollen (primäre Geldrollenaggregate) bei gleichzeitiger Abnahme der Zahl der Einzelerythrozyten. Wird die Konzentration hochmolekularer Substanzen (z. B. Fibrinogen) weiter erhöht, dann nimmt die Länge der primären Geldrollenaggregate (rouleaux) zu und die einzelnen Erythrozyten in den Geldrollen rücken näher zusammen. Schließlich bilden die primären Geldrollenaggregate untereinander durch wahllose Zusammenlagerung zu größeren Einheiten sogenannte sekundäre Geldrollenaggregate (s. Abb. 1).

Eine Trennung der Erythrozytenaggregate in Einzelerythrozyten (Desaggregation) kann auf verschiedenen Wegen erreicht werden. Sie geschieht einmal bei erhöhter Strömungsgeschwindigkeit durch die dabei auftretenden stärkeren Scherkräfte. (Andererseits begünstigt eine Strömungsverlangsamung mit Verminderung der *vis a tergo* das Auftreten von Erythrozytenaggregaten.) Weiter kann durch Änderung des osmotischen Druckes im umgebenden Plasma (s. Abb 4 und 5) eine Desaggregierung bewirkt werden und schließlich auch durch Zusatz verschiedener Substanzen zu Blut. Von makromolekularen Stoffen ist nach Infusion von niedrigviskösem Dextran (Rheomacrodex 10%) eine desaggregierende Wirkung beim Sludge-Phänomen beobachtet worden (5 15) Eine aggregathemmende Eigenschaft des Anti Malaria Mittels Hydrochloroquin wird beschrieben (16) Von niedermolekularen Substanzen wirken offensichtlich eine Reihe von oberflächenaktiven Stoffen Besonders ausgeprägt ist die Desaggregation durch UFS die deshalb speziell untersucht wurden (14 17) aber grundsätzlich gleichartig wirken auch Detergentien ($C_{12}$-sulfonat, Cetyltrimethylammoniumbromid) Eine «Anti Sludge»-Wirkung von Lezithin wird beschrieben (18) sie konnte aber von uns bei Untersuchungen mit gereinigtem Lezithin nicht bestätigt werden* Bei der Untersuchung anderer oberflächenaktiver Stoffe, wie des Aescins als Vertreter der Saponine und des Na-desoxycholats als Beispiel für Gallensäuren konnte im Gegensatz zu den vorher beschriebenen Detergentien kein Einfluß auf die Blutsenkung und keine desaggregierende Wirkung beobachtet werden. Bei Herzglykosiden fanden sich eine angedeutete Desaggregation und eine Viskositätserniedrigung (19) Die unterschiedliche Wirkung verschiedener oberflächenaktiver Stoffe läßt vermuten, daß bei der Gruppe der stark wirksamen Substanzen (Fettsäuren, Dodekansulfonat, Cetylmethylammoniumbromid) die gleichen chemischen Strukturen (langgliedriger Alkylrest in Verbindung mit einer hydrophilen polaren Gruppe) für die desaggregierende Wirkung wesentlich sind, wobei die Ladung der hydrophilen Gruppe offenbar nicht entscheidend ist. Oberflächenaktive Substanzen anderer chemischer Struktur wie Gallensäuren oder Saponine scheinen dagegen unwirksam zu sein. In dieses Schema würde sich auch die Beobachtung von Litschel und Tomcsik (20) einreihen, die eine desaggregierende Wirkung von Lysolezithin auf Geldrollen im Rinderblut beschrieben.

* Nicht veröffentlichte Beobachtung

In den vorliegenden Untersuchungen wurde die Desaggregation an Hand von Blutausstrichen, der Hemmung der Blutsenkung und des Verhaltens der Viskosität untersucht und beurteilt. Zusatz von oberflächenaktiven Substanzen, z.B. von Na-Oleat bewirkt eine Desaggregation der einzelnen Geldrollenaggregate und führt schließlich zum Auftreten von stechapfelförmigen Erythrozyten (Abb. 1 und 2). Diese Desaggregation kommt dadurch zustande, daß die Erythrozyten unter Abnahme ihres Durchmessers immer kugeliger werden (12) und dadurch schon rein mechanisch nicht mehr so eng aneinander liegen können als die flachen, scheibenförmigen Erythrozyten der Geldrollenaggregate. Als Ursache dieser Formveränderung der Erythrozyten ist eine verminderte Oberflächenspannung des Systems anzunehmen.

Der zunehmenden Sphärozytose entspricht eine geringgradige Zunahme des HT bei hohen Na-oleat Konzentrationen.

Die Abnahme der Viskosität eines aggregatreichen Blutes durch Zusatz von kleinen Mengen Na-oleat weist ebenfalls darauf hin, daß eine Desaggregation eingetreten ist (s. Abb. 3). Die größen und zahlenmäßig verkleinerten Erythrozytenaggregate passieren die dünne Kapillare des Viskosimeters leichter und schneller. Bei hohen Dosen von Oleat jedoch, wenn mikroskopisch keine Aggregate mehr zu sehen sind, steigt die Viskosität wieder deutlich an (Abb. 3). In den angegebenen Konzentrationsbereichen bleibt dabei die Plasmaviskosität konstant (17).

Ähnliche Verhältnisse ergeben sich auch beim Zusatz hypotoner Kochsalzlösungen. Durch mäßige Verringerung des osmotischen Druckes nimmt die Viskosität infolge der Desaggregierung vorhandener Aggregate leicht ab. Eine weitere Senkung des osmotischen Druckes führt zu einem Anstieg der Viskosität und des Hämatokrits und ist wohl auf die Volumenzunahme der Einzelerythrozyten zurückzuführen (Abb. 5). Noch auffälliger sind die Verhältnisse bei Glukose Zusätzen. Obwohl bei geringgradig hypertonen Konzentrationen der HT Wert absinkt und eine Desaggregation sichtbar wird, erhöht sich die Blutviskosität (Abb. 4).

Aus diesen Befunden ergibt sich, daß die Viskositätsänderungen nach Zusatz der oben angegebenen Substanzen als Wirkung sowohl auf die Erythrozytenaggregate (Desaggregation) als auch auf die Einzelerythrozyten (Formveränderung) zu erklären ist. Während niedrige Konzentrationen von Detergentien (z.B. Na-oleat bis etwa

30 mg%) oder geringe Veränderungen des osmotischen Druckes bei nur mäßigen Formveränderungen der Erythrozyten (Verkleinerung des Durchmessers, beginnende Sphärozytose) zu einer Viskositätsverminderung durch Desaggregation führen, bewirken hohe Konzentrationen von Oleat oder stärker hypotone Lösungen eine Erhöhung der Viskosität, als deren Ursache stärkergradige Formveränderungen der Einzelerythrozyten (Stechäpfel, Sphärozyten) anzunehmen sind. Theoretisch könnte die Viskositätserhöhung auch so gedeutet werden, daß es zur Bildung äußerst kurzlebiger Kleinstaggregate kommt, wobei im Sinne eines Fließgleichgewichtes eine kontinuierliche Aggregation und Desaggregation stattfindet.

Parallel zur Desaggregation von Geldrollenaggregaten nimmt eine vorher erhöhte BSG ab (Abb 3) Bei hohen Dosen von Oleat wird die Blutsenkung vollständig gehemmt (14) Nach Bergenhem und Fåhraeus (6) *kommt die Blutsenkung durch die Bildung von Erythrozytenaggregaten in Form von Geldrollen zustande.* Wie aus Abb 1 und 3 zu ersehen ist, geht umgekehrt die Desaggregation von Erythrozyten mit einer Normalisierung der BSG einher Der hemmende und stabilisierende Einfluß der UFS auf die BSG dürfte demnach durch die gleichen Mechanismen bedingt sein, die zu einer Desaggregation von sludged-blood führen.

Auf Grund unserer Ergebnisse können wir die Annahme von Thorsen und Hint (5) und anderen (6) bestätigen, daß es sich beim sludged blood wie auch bei der Blutsenkung letztlich um Geldrollenaggregate handelt. Die einfache Geldrollenbildung möchten wir als primäre Geldrollenaggregate, die Zusammenlagerung von Geldrollen ihrerseits zu größeren Verbänden als sekundäre Geldrollenaggregate bezeichnen. Zwischen beiden dürfte es je nach Schwere des Krankheitsbildes, der BSG-Veränderung und dem Grad des kapillarmikroskopischen sludged blood-Effektes fließende Übergänge geben.

Die auffallend starke desaggregierende Wirkung von unveresterten Fettsäuren bei nur geringgradig erhöhter Gesamtkonzentration im Blut läßt vermuten daß dieser *in vivo* vorkommenden und metabolisch wichtigen Fettfraktion auch eine physiologische Funktion in bezug auf das Blut zukommt (Erhaltung der Suspensionsstabilität des Blutes, physiologische »Anti-Sludge«-Substanz) Dies ist um so wahrscheinlicher als die bei *in vitro* Versuchen wirksamen Konzentrationen von UFS auch *in vivo* vorkommen können (21)

Zur Klärung dieser Fragen sind weitere Untersuchungen vorgesehen.

Frau E. [illegible] danken wir für ihre wertvolle Mitarbeit.


## *Zusammenfassung*

Durch Zugabe von Fibrinogen oder hochviskösem Dextran zum Blut gesunder Probanden entstehen die gleichen Erythrocytenaggregate, die bei stark beschleunigter Blutsenkung und beim sludged-blood-Phänomen gefunden werden. Die Desaggregation von sludged-blood-Aggregaten durch oberflächenaktive Substanzen, insbesondere unveresterten Fettsäuren, konnte an Hand vergleichender Viskositäts- und BSG-Messungen gezeigt werden. Durch eine besondere Ausstrichtechnik läßt sich die Aggregation oder die Desaggregation von Erythrocyten mikroskopisch belegen. Es wird zwischen primären und sekundären Geldrollenaggregaten unterschieden. Die mögliche physiologische Bedeutung der unveresterten Fettsäuren als desaggregierende Substanz wird diskutiert.

## *Summary*

Human-fibrinogen or high-viscous dextran, added *in vitro* to blood of young, healthy persons, causes the same aggregates of red cells, which are seen in cases of high erythrocyte sedimentation rate or in the sludged-blood-phenomen. The desaggregation of sludged-blood aggregates by surface-active substances, in particular non esterified fatty acids, could be shown by ESR and viscosity-determinations. A special slide-preparation to demonstrate the aggregates microscopally is described. The difference is made between primary and secondary rouleaux-aggregates. The possible physiological importance of the non esterified fatty acids as desaggregating substances is discussed.

## *Résumé*

Les aggrégats d'érythrocytes qui se trouvent dans le phénomène du «sludged-blood» et lorsque la vitesse de sédimentation des érythrocytes est fortement augmentée, peuvent être reproduits par l'adjonction de fibrinogène et de dextran fortement visqueux au sang de jeunes personnes saines. La désaggrégation d'érythrocytes à l'aide de substances tensio-actives, spécialement d'acides gras non-estérifiés, peut être démontrée par la détermination comparée de la vitesse de sédimentation et de la viscosité. L'aggrégation et la désaggrégation d'érythrocytes peuvent être démontrées microscopiquement. Une différenciation est faite entre aggrégats en forme de rouleaux primaires et secondaires. L'importance physiologique possible des acides gras non-estérifiés, en tant que substances désaggrégeantes, est discutée.


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# The Transformation of Human Mononuclear Leukocytes in vitro

## I. Comparison of Cells in Suspension and Attached to Coverslips

J. O. Lamvik

In the last few years several investigators have clearly demonstrated that lymphocytes from human peripheral blood are able to transform to large, hyperchromatic blastoid cells when stimulated by Phytohaemagglutinin (18), different bacterial antigens (10) or homologous leukocytes (1).

In the older medical literature many reports are found about the transformation of lymphocytes into large, phagocytic mononuclear cells *in vitro* without special stimulation (2, 4, 6, 17, 20). The precursors of these large mononuclear cells, however, have been subject of controversy. Some investigators (12, 14, 16) have reported that only monocytes form macrophages, while others (5, 15) have attributed this capacity to both monocytes and lymphocytes. Rebuck *et al.* (19) have described the gradual change of lymphocytes to macrophages in supravital preparations of material taken from cantharides blisters on the human forearm, while Ebert *et al.* (9) and Gowans (1) watching rabbit and rat lymphocytes in fluid chambers did not find any transformation of small lymphocytes into other cells.

Timofejewski and Benewolenskaja (21) found cells in mitosis in leukocyte cultures and Chrustschoff (7) used leukocyte cultures with added embryo extract and lysate of blood clot to study the karyotype in dividing cells. Apart from these reports the papers describing the development of large mononuclear cells *in vitro* do not mention cell division. The large mononuclear cells are usually described as cells resembling macrophages with abundant cytoplasma and with phagocytic ability. The cells differ clearly from the blastoid cells in culture of stimulated lymphocytes, which resemble lymphoblasts or plasmablasts morphologically.

Thus, according to the medical literature, the small lymphocyte seems to be able to transform into two different types of cells, depending on the culture conditions.

The purpose of this investigation was to study the morphological changes in non-stimulated leukocytes cultures *in vitro*. The conditions were similar to those used for studies on blastoid transformation, but stimulating agents were not added. Several investigators describing the *in vitro* development of large mononuclear

cells have studied the cells attached to glass (3–6) while none have described this transformation conlusively in suspension cultures. Therefore leukocyte suspension cultures were prepared with cover slips applied to the culture tubes, making it possible to observe glass-attached cells and unattached cells coming from the same leukocyte inoculum.

In one part of the experiment the cells in suspension were studied after preparation of smears. This procedure brought the cells in close contact with the surface of the slide which could possibly change the morphological appearance of the cells. In the other part of the experiment, therefore, supravital staining with Acridine Orange was used on cells suspended in culture medium, giving the morphological appearance and the vitality of the cells in suspension. No attempt was made to evaluate the vitality of the cells attached to the coverslips.

## *Material and Methods*

Pyrex glass tubes, measuring 14 by 120 mm were used for the cell cultures. The tubes were washed in 0.5% Haemo-sol (Merz & Dade AG, Bern) thoroughly rinsed in tap water and distilled water dried at 40 °C, wrapped in paper and sterilized by dry heat at 160 °C.

Coverslips (change No. 1) measuring 11 by 22 mm were placed in 1% HCl for 2–24 h, rinsed in tap water washed in boiling 0.5% Haemo-sol for 5–10 min, again rinsed in tap water and transferred to small plastic racks. The racks were placed in warm Haemo-sol for 1 h, rinsed in tap water for 1–2 h, followed by several dips in distilled water. The racks were then dipped in 100% ethanol and in ether which caused rapid drying of the glass surfaces. Finally each dry coverslip was wrapped in thin aluminium foil and sterilized by dry heat.

Venous blood was obtained from a normal blood donor and mixed with 1 ml heparin, Evans (1000 i. u.) and 4 ml 6% dextran (Intradex., Nyco) per 15 ml of blood. The erythrocytes were separated by sedimentation for 45 min at 37 °C. The leukocyte and platelet rich plasma was transferred to siliconized tubes and centrifuged at 150 g for 10 min. The supernatant containing platelets and very few leucocytes was discarded. The leukocytes were gently suspended in autologous plasma. Cell counts and differential counts in prepared smears were estimated using the methods of Dacie ([illegible])

A series of culture tubes was prepared, each containing $2 \times 10^6$ leukocytes in 2.5 ml culture medium consisting of 25% fresh autologous plasma and 75 % Parker's tissue culture medium (TC 199) Difco at pH 7.2. A coverglass was placed in each tube. The tubes were stoppered by rubber stoppers and placed in specially made racks so that the coverslips had an inclination of 60° from the horizontal.

The culture tubes were harvested in duplicate at regular intervals. The coverslips were dipped in saline to remove unattached cells, then rapidly dried under a fan, fixed in absolute methanol and stained with May-Grünwald-Giemsa. The unattached cells were mixed by rotation of the tubes and the cell counts determined. The suspensions were then centrifuged at 500 g for 10 min and smears made, which were fixed and stained with May-Grünwald-Giemsa. Differential counts were carried out.

The cell number on the coverslips was estimated by counting the cells in 4 high power fields, each field having diameter of 0,4 mm. Four high power fields thus constituted 1/500 part of the coverslip. There was some variation in the cell number in different parts of the coverslips according to the depth of cell suspension over each part of them. Thus, one end of each coverslip was regularly more cellular then the other Because of this variation the cells were always counted in two fields in the middle of the coverslip and one field in each end. All cells counted were differentiated into granulocytes, lymphocytes or large mononuclear cells.

In the second part of the experiment the cell suspensions after mixing and estimation of cell numbers, were centrifuged t low speed (50 g) for 5 min to reduce the volume to 1/4 of the original and then mixed with an equal olume of Acridine Orange, 9 mg/100 ml TC 199 pH 7 4. After staining period of 10 one drop of the suspension was transferred to clean microscopic slide. A coverslip was applied the cells observed by fluorescent microscopy and differential counts estimated within 1 2 min. The microscope used was Zeiss photomicroscope with an Osram HBO 200 mercury vapor lamp as the light source. A Zeiss BG 12 exiter filter was used with yellow-orange absorption filter

In the supravitally stained preparations the differential counts were determined on cells surrounded by fluid, as none were found attached to the slides or the coverslips during the counting The cell vitality was evaluated from the nuclear fluorescence. At pH 7 4 vital cells display bright, apple-green nuclear fluorescence, while the nuclei of injured cells fluoresce red or with deep, dull-green stain (13) The cytoplasm of vital as well as injured cells shows orange or red fluorescence and is unreliable for differentiation between vital and damaged cells.

## *Results*

The variations in the numbers of unattached cells and cell numbers on the coverslips are given in Table I The table further shows the variations in the differential counts, estimated from May Grünwald-Giemsa stained smears of unattached cells and cover slip preparations of attached cells. Fig 1 shows the variations in the absolute numbers of the different cell types.

The number of granulocytes dropped rapidly on the cover slips as well as in suspension At the same time there was an increase in the number of damaged, unattached cells. There was a marked difference between the attached and unattached cell populations in the proportions of small and large mononuclear cells. On the coverslips large mononuclear cells were numerous after 3 h culture and were the predominating cell type on the third day Fig 2) while lymphocytes were scanty throughout. Among unattached cells small lymphocytes were present in high and steady number throughout, apart from a drop after a 3 h culture time possibly due to transient attachment. A few large mononuclear cells were found in the smears of unattached cells, mainly in the end of the preparations.

*Table I*

Cell numbers and differential counts in leukocyte cultures (mean values from duplicate culture tubes)

| | Culture time h | Cell No. | Differential counts (%) | | | |
|---|---|---|---|---|---|---|
| | | | granulocytes | lymphocytes | large mononucl. cells | damaged cells |
| Unattached cells | 0 | 600 | 70 | 19 | 5 | 6 |
| | 3 | 455 | 70 | 10 | 9 | 11 |
| | 15 | 360 | 34 | 30 | 3 | 33 |
| | 40 | 430 | 29 | 49 | 3 | 18 |
| | 65 | 335 | 30 | 26 | 8 | 36 |
| Attached cells | 3 | 375 | 74 | 5 | 17 | 4 |
| | 15 | 331 | 66 | 9 | 22 | 4 |
| | 40 | 236 | 50 | 13 | 32 | 5 |
| | 65 | 120 | 6 | 7 | 85 | 2 |

Cell number per mm³ in suspension.
Cell number in 4 high power fields on coverslip.

To investigate the possibility that these large cells obtained their appearance because of contact with the glass surface before the smears had dried another series of culture tubes with cover slips were prepared as in the preceding experiment. The unattached cells were examined by fluorescence microscopy after Acridine Orange staining as described. In Table II the variations in the cell number and differential counts of vital and non vital unattached cells are given. No large mononuclear cells were found after Acridine Orange supravital staining The number of vital granulocytes showed a gradual decline while the absolute number of vital lymphocytes did not change significantly On the coverslips the results were as in the preceding experiment. The large mononuclear cell was the predominant cell type after a 48 h culture period.

## *Discussion*

No transformation of lymphocytes into large mononuclear cells or blastoid cells was noticed in non-stimulated leukocyte suspension cultures. The lack of transformation in the fluid phase was not due to loss of cell vitality as the majority of the lymphocytes were vital as judged by Acridine Orange supravital staining Furthermore the culture medium used has been found sufficient for marked blastoid transformation of lymphocytes on the addition of

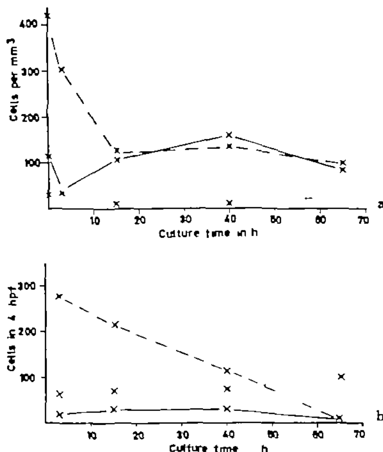



*Fig. 1* Variations in the numbers of unattached cells ( ) and cell numbers per 4 high power fields on coverslips (b) in leukocyte cultures. Each mark is the mean of the values from duplicate culture tubes. Granulocytes = — — — lymphocytes = —— large mononuclear cells =

Phytohaemagglutinin. The findings are in accordance with GOWANS (11) who found no transformation of rat thoracic duct lymphocytes in fluid chambers. Most of the earlier reports about lymphocyte or monocyte transformation to large mononuclear cells have described cells in hanging drop cultures, where the cells are observed in a plasma clot or attached to a coverslip surface (2, 4 6, 15 17 20) The present findings also show that leukocytes can change to large mononuclear cells when attached to a glass sur face. Possibly those mononuclear cells which are able to stick to a glass surface, change to large mononuclear cells when they spread

*Table II*

Cell numbers, differential counts and vitality of unattached cells in leukocyte cultures (mean values from duplicate culture tubes)

| | | Differential counts (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | vital cells | | | non-vital cells | | |
| Culture time h | Cell No. per mm³ | granulocytes | lymphocytes | large mononucl. cells | granulocytes | lymphocytes | large mononucl. cells |
| 0 | 700 | 48 | 52 | 0 | 0 | 0 | 0 |
| 5 | 605 | 51 | 47 | 0 | 2 | 0 | 0 |
| 24 | 630 | 48 | 48 | 0 | 3 | 1 | 0 |
| 48 | 570 | 18 | 60 | 0 | 17 | 5 | 0 |
| 72 | 530 | 13 | 39 | 0 | 39 | 9 | 0 |
| 96 | 530 | 10 | 39 | 0 | 39 | 12 | 0 |

on the glass. A similar mechanism may also explain the development of large mononuclear cells in plasma clot cultures where cells may adhere to and spread on fibrin threads. Another possibility is that stimulating agents may be present in the culture medium and cause blastoid transformation. This is the most likely explanation for the dividing cells found by CHRUSTSCHOFF (7)

Most of the large cells found on the coverslips in the present experiment had round or oval, leptochromatic nuclei and sky blue, vacuolated cytoplasm, often containing engulfed material On some coverslips the predominant cell type was shaped like fibroplasts with long cytoplasmatic projections. These cells were found in varying numbers on all coverslips and were sometimes found in greater numbers on one of two duplicate coverslips the other one showing predominantly the round mononuclear cells. *Thus it seems probable that the shape of the cells is determined to* some extent by the quality of the glass surface. Possibly the development of fibroblast like cells is due to an uneven glass surface or the presence of deposits which prevent the cells from spreading regularly

The culture experiments gave no evidence as to the precoursors of the large mononuclear cells. The granulocytes which stick to the glass easily show degenerative changes early and can be excluded as possible precoursors. Some lymphocytes were found on the coverslips, but the majority of the small lymphocytes appeared to be non sticky No evidence of transformation between lymphocytes and large mononuclear *cells* was *found. The monocyte has* been considered the most probable precursor of the large mononuclear cells (22) The monocytes in the cell suspensions were



*Fig. 2.* Large mononuclear cells in leukocyte coverslip culture after 65 h culture. May-Grünwald-Giemsa, ×420

however, very scanty and hardly numerous enough to act as precursors for all the mononuclear cells on the coverslips. Further experiments will be reported on the relationship between leukocytes and large mononuclear cells.

## *Summary*

Human leukocytes were cultured in fluid medium in Pyrex tubes containing coverslips. The appearance of non-stimulated cells attached to glass was compared with unattached cells in the fluid medium. The granulocytes showed degenerative changes and gradually became reduced in number. Small lymphocytes were predominant in the fluid medium and few attached to the coverslips. Large mononuclear cells were numerous on the coverslips, while none were found in the fluid phase. These cells probably attain their appearance on contact with the glass surface. They are clearly different from the lymphoblastoid cells found in cultures of stimulated lymphocytes. No transition was noticed in early coverslip cultures between lymphocytes and large mononuclear cells.

## *Zusammenfassung*

Menschliche Leukozyten wurden in flüssigem Medium kultiviert in Pyrex-Röhrchen, die Deckgläschen enthielten. Es wurde verglichen zwischen den am Glas haftenden, nicht stimulierten Zellen und den nicht haftenden Zellen des Mediums. Die Gra-

nulocyten zeigten degenerative Veränderungen und eine *fortschreitende Abnahme ihrer* Zahl. Die kleinen Lymphocyten waren im flüssigen Medium vorherrschend und nur wenige hafteten an den Deckgläschen. Grosse mononukleäre Zellen fanden sich reichlich an den Deckgläschen, nicht jedoch in der flüssigen Phase. Wahrscheinlich nehmen diese Zellen ihre Gestalt beim Kontakt mit der Glasoberfläche an. Sie unterscheiden sich deutlich von lymphoblastenähnlichen Zellen in Kulturen stimulierter Lymphocyten. I  früben Deckglaskulturen fand sich kein Übergang zwischen Lymphocyten und grossen mononukleären Zellen.

## *Résumé*

Des leucocytes humains ont été cultivés dans un milieu fluide dans des tubes Pyrex contenant des couvre-objets. L aspect des cellules non-stimulées fixées au verre été comparé à celui des cellules non-fixées se trouvant dans le milieu fluide. Les granulocytes présentèrent des altérations dégénératives et diminuèrent progressivement en nombre. Les petits lymphocytes prédominèrent dans le milieu fluide et peu d'entre eux se fixèrent aux couvre-objets. Les grandes cellules mononucléaires étaient fixées en grand nombre aux couvre-objets et aucune ne fut trouvée dans la phase fluide Ces cellules prennent probablement leur aspect contact de la surface de verre. Elles sont de façon très distincte différentes des cellules lymphoblastoïdes trouvées dans les cultures lymphocytes stimulés. *Aucune forme de transition ne fut observée dans les cultures contenant* des couvre-objets entre lymphocytes et grandes cellules mononucléaires.

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# Studies of Lymphocytes and Their Derivative Cells in vitro

## I. Biochemical Constituents

J. Gough* and M. W. Elves

When lymphocytes from human blood are incubated *in vitro* in the presence of phytohaemagglutinin (PHA) the majority transform into larger nucleolated pyroninophilic cells (4, 6, 15). Macrophages are also formed when leucocyte suspensions are incubated, and it has recently been shown that it is the lymphocytes which give rise to the majority of these cells (10). In the present study cytochemical methods have been used to compare and contrast the biochemical constituents of the PHA transformed cell, the macrophage and their precursor cell, the lymphocyte.

### Methods

*Cell Cultures*

Leucocyte cultures were prepared using normal human blood by the method of Hungerford *et al.* (11) except that PHA was replaced as a separating agent by [illegible] ml of 6% solution of dextran in isotonic saline ("Dextraven", Benger Ltd.) per 20 ml of blood. 2–3 ml of the resulting cell suspension in plasma was made up to 10 ml with TC 199 (Glaxo) and incubated at 37 °C in 25 ml universal containers for various times. 0.05 ml of PHA-P (Difco) was added to some cultures. Macrophages were examined from cultures untreated with PHA by centrifuging for 5 min at 500 rpm after 48 h incubation, resuspending the cells in a drop of fresh serum, and making smears. Smears of PHA-treated cultures were prepared in the same way after 72 h incubation. Smears of buffy coats were used to study untransformed leucocytes.

*Cytochemical Procedures*

*Methyl green-pyronin.* The standard method evolved by Brachet (3) and modified by Kurnick (13) was used.

*Mercury-bromphenol blue.* The method is based on that of Mazia *et al.* (17) using films fixed in Carnoy's solution.

*DDD reaction (sulphydryl groups).* The method used was that described by Pearse (18) which is based on that of Barrnett and Seligman (2).

*Periodic acid-Schiff.* The standard method (16) using formalin vapour fixation.

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* The contribution by this author formed part of an M.D. thesis accepted by the University of Cambridge.

*Sudan black B.* The method used was that of SHEEHAN AND STOREY (21) using formalin vapour fixation, staining time of 30 min and Giemsa counterstain.

*Feulgen.* The method described by PEARSE was used (18). Control slides were treated with xylene immediately after fixation.

## Results

*Methyl green-pyronin.* All cell nuclei gave a positive reaction for DNA, while the cytoplasm of lymphocytes, polymorphs and monocytes were found to contain varying amounts of RNA. The intensity of staining of the cytoplasm of macrophages was about the same as that of untransformed lymphocytes vacuoles in the macrophages were unstained. In PHA transformed cells striking changes were observed. In the intermediate type of cell an RNA positive nucleolus was generally present in the nucleus and there was an increase in the amount of pyroninophilia in the cytoplasm compared with the small lymphocyte. The 'blast'-like cell contained one or more nucleoli, and the cytoplasm gave a strongly positive reaction for RNA the cytoplasmic vacuoles were clear. These results indicate that the RNA content increases progressively as lymphocytes transform under the influence of PHA to an intermediate and finally a 'blast'-like cell which contains a considerable amount of RNA.

*Mercury-bromphenol blue.* The nuclei and cytoplasm of all the cells studied gave a positive reaction of varying degrees. The nuclei were more deeply stained than the cytoplasm. The cytoplasm of lymphocytes and polymorphs was weakly positive. In macrophages the vacuoles remained unstained, and were surrounded by pale-staining cytoplasm. The cytoplasm of PHA transformed cells stained more deeply and the vacuoles in the 'blast' like cells were clear.

*DDD reaction.* All cells gave a red positive reaction, the cytoplasm generally staining deeper than the nuclei, indicating that nuclear and cytoplasmic protein of all the cell types studied contain SH groups. The specific granules of neutrophils were often prominently stained.

*Periodic acid-Schiff.* Polymorphs from buffy coat smears were shown to contain glycogen, exhibiting a strongly positive staining reaction while control slides incubated with salivary amylase were negative. Most lymphocytes were negative, but a small proportion

of cells had a few discrete granules, against a negative cytoplasmic background These granules were unstained in control smears.

Lymphocytes from cell cultures untreated with PHA had similar features to those from buffy coats. The positivity of polymorphs was markedly reduced. Some macrophages were negative, but some possessed a diffuse positivity which was often perivacuolar in distribution. No blocks of glycogen were seen, and the positivity was not seen in saliva-treated control smears. The vacuoles were negative.

In PHA transformed cells large blocks of positivity were seen especially in the 'intermediate cells. Most blast-like cells were negative but a number possessed small blocks of glycogen. In PHA treated cultures 40–50 / of lymphoid cells were PAS positive after 24 h incubation, and 60–70 / after 72 h incubation In these cultures some small lymphocytes had small blocks of glycogen.

*Sudan black B* Polymorphs from buffy coats were all strongly positive, and a granular pattern was often discernible. Monocytes were often positive, but less strongly so and exhibited a discrete granular reaction. Lymphocytes were invariably negative.

In PHA treated cultures the polymorphs and lymphocytes presented the same reactions as in buffy coat smears. The PHA transformed cells were negative.

In cultures untreated with PHA polymorphs were again positive, but where degenerate their vacuoles were negative. Most macrophages were positive to a varying extent, with an occasional negative cell. Vacuoles in macrophages were generally negative. Some macrophages contained blocks of intense sudanophilia suggesting that this is derived from phagocytosed polymorph material.

Xylene extraction for one hour did not influence these reactions indicating that sudanophilia is not due to free neutral lipid

*Fettrot.* Polymorphs from buffy coats were negative while lymphocytes contained an occasional positive granule.

The cytoplasm of PHA transformed cells was negative, but 1–3 positive vacuoles were seen in many cells most of the vacuoles were negative. Vacuolar positivity was shown to be due to neutral fat by its removal after exposure to xylene.

Macrophages were seen to possess negative cytoplasm but the vacuoles were usually positive to varying degrees. Positivity in these cells was also removed by xylene.

*Discussion*

The increase in pyroninophilia which occurs when a small lymphocyte transforms in the presence of PHA indicates that a considerable degree of RNA synthesis must have occurred. This has been confirmed by McIntyre and Ebaugh (14) who found a marked increase in RNA synthesis in the first 24 h of culture in the presence and absence of PHA, but as time progressed PHA treated cultures demonstrated a higher rate of RNA synthesis. Epstein and Stohlman (7) demonstrated a marked uptake of tritiated cytidine by lymphoid cells in the first 24 h of culture in the presence of PHA, but little uptake in control cultures. No DNA synthesis occurs in the early cultural period so this uptake indicated RNA synthesis.

The presence of an increased protein content of PHA transformed cells compared with lymphocytes similarly indicates that protein synthesis has occurred. This correlates with the finding of increased amounts of ribosomes and endoplasmic reticulum in these cells (5 12, 22) and an increase in glutamic dehydrogenase activity which links protein and carbohydrate metabolism (9)

The presence of large amounts of glycogen in neutrophils and primitive myeloid cells, and small amounts in lymphocytes was demonstrated by Gibb and Stowell (8) The same results have been obtained in the present study and confirmed by others (1)

In PHA-treated cultures many intermediate cells were strongly positive for glycogen, whereas most 'blast -like cells were negative similar results were obtained by Quaglino *et al.* (19) This suggests that as transformation proceeds the lymphocyte synthesizes a store of glycogen which is metabolised as the 'blast stage is reached.

The moderate degree of PAS positivity which was seen in a small number of macrophages may have been due to ingested polymorph debris. In macrophages obtained by the skin window technique of Rebuck and Crowley (20) the amount of glycogen increased as the inflammatory reaction proceeded (23)

The strong sudanophilia of neutrophils is well known and the presence of blocks of intense sudanophilia in some macrophages again suggests a derivation from phagocytosed polymorph debris.

There is no sudanophilia in PHA-transformed cells, but the presence of Fettrot positive vacuoles indicates neutral fat, which is removed by alcohol in the Sudan black method. It has been suggested that the clear vacuoles seen in these cells in Romanowsky

stained smears may correspond to the dense osmiophilic bodies found in these cells when examined with the electron microscope (5).

Thus the two lymphocytic transformations are accompanied by changes in the biochemical constitution of the cells. The PHA stimulated transformation is accompanied by the greater synthetic activity while many of the changes seen in the macrophages may be due to their phagocytic properties.

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## *Summary*

Lymphocytes, PHA-transformed cells and macrophages derived from *in vitro* leucocyte cultures have been examined cytochemically for certain biochemical constituents. PHA-transformed cells contain increased amounts of protein, RNA and glycogen compared with the lymphocyte, except at the blastoid stage when glycogen is often absent; some of the cytoplasmic vacuoles of these cells are lipid positive. Macrophages do not show any increase of protein or RNA compared with the lymphocyte; some cells contain glycogen and most cytoplasmic vacuoles contain neutral lipid.

## *Zusammenfassung*

Lymphocyten, durch Phytohämagglutinin (PHA) umgewandelte Zellen und Makrophagen aus Leukocytenkulturen *in vitro* wurden histochemisch auf bestimmte biochemische Bestandteile untersucht. Im Vergleich zu Lymphocyten zeigen die durch PHA umgewandelten Zellen einen erhöhten Gehalt an Eiweiss, RNA und Glykogen, mit Ausnahme blastoider Vorstufen, die oft glykogenfrei sind. Einige Zytoplasmavakuolen dieser Zellen geben eine positive Lipidreaktion. Makrophagen zeigen im Vergleich zu Lymphocyten keine Zunahme von Eiweiss oder RNA; einige Zellen enthalten Glykogen und die meisten Zytoplasmavakuolen enthalten Neutralfette.

## *Résumé*

Certains constituants biochimiques de lymphocytes, de cellules transformées à l'aide de PHA et de macrophages dérivés de cultures de leucocytes ont été étudiés à l'aide de méthodes cytochimiques. Les cellules transformées à l'aide de PHA contiennent des quantités augmentées de protéines, d'ARN et de glycogène comparées aux lymphocytes, à l'exception du stade blastoïde dans lequel le glycogène est souvent absent. Certaines vacuoles cytoplasmatiques de ces cellules donnent une réaction positive aux colorants de lipides. Les macrophages ne montrent aucune augmentation de leur contenu en protéines ou en ARN en comparaison aux lymphocytes; quelques cellules contiennent du glycogène et la plupart des vacuoles des graisses neutres.

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# Eosinophil Granules and Cell Maturity: Electron Microscopic Observations on Guinea Pig Marrow

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The specific granules of the eosinophil leucocyte have a striking appearance in the electron microscope. They are usually described as having a limiting membrane enclosing an outer portion or matrix, in the centre of which is an inner part, variously referred to as the internum (3, 11, 17), the inclusion (8), the crystalloid or the crystal (4, 6, 7). In bone marrow eosinophils, other granules have been observed which show no internum on section (4, 6, 7, 19, 20) and it is usually suggested that these should be regarded as immature granules. However, a definite relationship between the two types of granule as seen in the electron microscope does not seem to have been established. It therefore appeared worth while to attempt a comparison of the relative numbers and dimensions of the profiles of these granules at various developmental stages in the bone marrow of the guinea pig. It was hoped that such information might throw light on the significance of the two varieties of granule and on changes which take place during cell maturation. This communication is based upon the results obtained.

## *Material and Methods*

The observations were made on bone marrow obtained from 10 male guinea-pigs of the Dunkin-Hartley strain. Each weighed approximately 400 g at the time of examination. Such animals are used as the standard for quantitative haematological observations in this laboratory (18, 22). Five of the animals were completely normal and 5 had each received 4 subcutaneous injections of 1 ml horse serum at 5-day intervals, the bone marrow being examined 3 days after the last injection. As expected from previous

studies of the marrow eosinophils in the foreign protein response (15, 16) early forms of eosinophil were subsequently observed much more readily in the bone marrow of the animals which had received foreign protein. This treatment did not seem to interfere with the general well-being of the animal and there was no sign of anaphylactic or other reaction.

T obtain tissue for electron microscopy the animal was killed with a blow Bone marrow was rapidly removed from one femur and specimens were fixed for 2.5 h in 1% osmium tetroxide buffered at pH 7.3. After washing, the specimens were taken through increasing strengths of alcohol. Staining was carried out at the end of dehydration with a 1 percent solution of phosphotungstic acid in absolute alcohol, for periods varying from 0.5 to 4 h. This method of staining was both very satisfactory and well-suited to the purpose, in that in all the subsequent micrographs, the interna of the eosinophil granules stand out as relatively electron transparent areas in the centre of dense matrix (17) It should be noted that freshly-prepared staining solutions were used and that the specimen blocks were of small dimensions (not more than 1 mm³) both these points are thought to be important in obtaining consistent staining with this method. Following staining, the blocks were embedded in araldite and sections were cut with glass knives made with an LKB knifemaker The sections were examined with Hitachi HS7 electron microscope.

Micrographs of nucleated cells containing typical eosinophil granules were obtained at instrument magnifications of between 5000 and 10000 times. Where possible the micrographs were classified into three developmental categories, namely 'myelocytes 'metamyelocytes' and 'band and segmented forms Classification was based on the ultrastructural observations of [illegible] (6) considered in relation to conventional light microscope criteria (21) However the granules were completely disregarded for purposes of classification. Two points to which particular attention was paid were the morphology of the nucleus and the degree of development of the endoplasmic reticulum. The nucleus of the 'myelocyte' was typically round or oval in section, with fairly smooth contour The nucleus of the 'metamyelocyte' sometimes had an indented outline (kidney-shaped) and sometimes its contour was irregular The 'band and segmented forms appeared in section to have more than one lobe to the nucleus, although of course, the 'lobes may have been part of a single deformed nucleus. An occasional myelocyte' showed evidence of nucleolus and one eosinophil in mitosis was included in this category [It is usually stated that cell division does not occur after the myelocyte stage (9)]. The membranous element of the endoplasmic reticulum with its associated particles was usually well-developed in the cytoplasm of the 'myelocytes' In the 'band and segmented forms there was little or no evidence of this membranous element, while the cells classified as metamyelocytes usually had moderate amount.

I all, 75 micrographs were studied, 25 from each developmental category In each category 10 micrographs were obtained from the 5 normal animals and 15 were obtained from the 5 animals in which eosinopoiesis had been stimulated. In each micrograph, all the granules with one diameter exceeding 0.2 μ were counted and classified as either showing or not showing evidence of an internum, with its typically straight sides and sharp corners. The total number of granules studied was 2396 representing an average of 32 per micrograph. The corresponding figures for each cell category were: myelocytes, 919 and 37; metamyelocytes, 766 and 31; band and segmented, 711 and 28 total normal, 973 and 32 total stimulated, 1423 and 32. In each micrograph, the profile of the internum-containing granule with the largest single diameter was then identified. This diameter (length) was measured, and the greatest dimension of the same granule-profile in plane at right angles was also determined (breadth) Similarly for the granules showing no internum, the profile with the largest single dimension was identified and measured in exactly the same way

*Results*

*Percentage of granules showing an internum.* It may be seen from Table I that the granules of the band and segmented forms showed an internum in a much higher percentage of cases than did those of the earlier forms. This highly significant difference is illustrated by representative cells in Fig 1 and 2 There is also a difference in Table I between the results for the myelocytes and those for the metamyelocytes and this is significant at the 0 05 level of probability when the total figures are considered. The differences between the results obtained from the normal and those obtained from the stimulated marrows were not statistically significant.

*Dimensions of the granule profiles* The average dimensions of the largest granule profiles are shown in Table II There are no statistically significant differences between the three developmental categories (A, B and C) with respect to the internum-containing granules. The profiles of the granules without interna were however much smaller both in length and breadth in the band and segmented forms than in the myelocytes and metamyelocytes. The differences are highly significant.

It may also be seen from Table II that the length of the internum-containing profiles was on average somewhat greater in the stimulated than in the normal marrow and this difference is reflected in a somewhat larger value for length breadth. Both these differences are significant at the 0 05 level but not at the 0 01 level of probability There are no significant differences between normal and stimulated marrow with respect to the granules without interna.

The average values for length breadth for the profiles of the granules with and without interna respectively may now be compared It can be seen in Table II that the profiles of the granules with interna had greater average values for length breadth i e. they were more ovoid There is a highly significant difference between the values obtained for the two types of granule in each of the various categories (A–F) The t values are as follows myelocytes

*Fig* A band or segmented eosinophil from the same section as Fig 1 I this cell, seen at a somewhat lower magnification, the majority of the granules show evidence of an internum. Part of an early eosinophil has also been included in the upper left part of this micrograph, and in its cytoplasm one can see the membranous element of the endoplasmic reticulum, as well as numerous granules showing no evidence of an internum. (× 11000)




*Fig. 1.* Eosinophil myelocyte in normal marrow (phosphotungstic acid staining). Granules such as G1 show a relatively pale internum in the centre of an electron-dense matrix. Other granules such as G2 show no sign of an internum. Note also that G1 appears ovoid and that the long axis of the internum corresponds to that of the granule profile, whereas G2 is more rounded. Some granules seem to have more than one internum. The granules are readily distinguished from mitochondria (M). The membranous element of the endoplasmic reticulum (ER) may be noted. (13900)

6.9 metamyelocytes 3.3 band and segmented forms 4.5 total normal 4.5 total stimulated 7.7 grand total 8.5. The difference can also be visualized in Fig. 1 by comparing for example the granules labelled 'G1' and 'G2'.

*Other observations.* Of all the granules showing an internum approximately 10% showed evidence of either more than one internum or a split internum. Examples may be seen in Fig. 1 and 2. The numbers were too small to warrant firm conclusions, but the impression gained was that there was no difference either between

*Table I*
Percentage of granules showing an internum

| | Normal | Stimulated | Total |
|---|---|---|---|
| A. Myelocytes | 48.7 ± 16.4 | 44.5 ± 15.6 | 46.2 ± 15.7 |
| B. Metamyelocytes | 60.5 ± 14.6 | 54.4 ± 16.0 | 56.9 ± 15.2 |
| C. Band and segmented forms | 82.1 ± 15.2 | 75.1 ± 12.1 | 77.9 ± 13.6 |
| t values: | | | |
| A–B | 1.7 | 1.7 | 2.4 |
| B–C | 3.2 | 4.0 | 5.2 |
| A–C | 4.7 | 6.0 | 7.6 |

*Average values ± standard deviations.* The t values are those of the standard error test for small samples and were calculated following the procedure given by Hudson (13). The t values exceeding 2.1 may be taken to indicate significance at the 0.05 level of probability, those exceeding 2.9 similarly indicating significance at the 0.01 level.

*Table II*
Dimensions of granule profiles

| | Granules with interna | | | Granules without interna | | |
|---|---|---|---|---|---|---|
| | Length | Breadth | Length/breadth | Length | Breadth | Length/breadth |
| A. Myelocytes | 0.89 ± 0.18 | 0.47 ± 0.13 | 2.0 ± 0.5 | 0.81 ± 0.13 | 0.67 ± 0.10 | 1.3 ± 0.6 |
| B. Metamyelocytes | 0.89 ± 0.19 | 0.52 ± 0.15 | 1.8 ± 0.6 | 0.85 ± 0.18 | 0.63 ± 0.16 | 1.4 ± 0.3 |
| C. Band and segmented forms | 0.98 ± 0.20 | 0.48 ± 0.14 | 2.1 ± 0.6 | 0.64 ± 0.22 | 0.43 ± 0.15 | 1.5 ± 0.3 |
| D. Total normal | 0.86 ± 0.22 | 0.50 ± 0.15 | 1.8 ± 0.5 | 0.72 ± 0.20 | 0.56 ± 0.17 | 1.3 ± 0.3 |
| E. Total stimulated | 0.96 ± 0.16 | 0.49 ± 0.12 | 2.1 ± 0.5 | 0.81 ± 0.22 | 0.59 ± 0.18 | 1.4 ± 0.3 |
| F. Grand total | 0.92 ± 0.19 | 0.49 ± 0.14 | 2.0 ± 0.5 | 0.78 ± 0.20 | 0.58 ± 0.17 | 1.4 ± 0.3 |
| t values: | | | | | | |
| A–B | 0.0 | 1.3 | 1.4 | 0.2 | 1.0 | 1.4 |
| B–C | 1.6 | 1.0 | 1.9 | 3.7 | 4.5 | 1.2 |
| A–C | 1.7 | 0.3 | 0.7 | 3.9 | 6.6 | 3 |
| D–E | 3 | 0.3 | 4 | 1.8 | 0.7 | 1.4 |

See footnotes to Table I

the various categories of cell or between the normal and stimulated marrows, with respect to the frequency with which this appearance was observed.

### *Discussion*

*The internum and cell maturity* Other investigators have drawn attention to the fact that in the immature forms of eosinophil, one frequently sees granules which show no evidence of an internum on section (4 6 7 8 19) The present observations confirm this and in addition give some indication of the actual frequency with which granule-sections show an internum at different developmental stages. It must be emphasized that the figures obtained in Table I do not represent the percentages of complete granules possessing an internum. They only refer to the slices of eosinophil granules which are obtained by ultra thin sectioning of the tissue. Apart from possible errors in classifying cells and in detecting the presence of an internum, one must bear in mind that a granule which shows no evidence on an internum in one section may well show such evidence in a different section. Nevertheless, a comparison of the figures in each category is of value in indicating that the more mature the cell, the greater is the proportion of granules which shown an internum in any particular section. While this would seem to suggest corresponding changes in the relative numbers of the two types of granule during the course of cell maturation, possible changes in the relative size of the granules must also be con sidered.

*Significance of the measurements of dimensions* It was in an attempt to obtain some indication of granule size and shape that the di mensions of the largest granule profiles (with and without interna respectively) were measured in each micrograph. It seemed rea sonable to suppose that, provided an adequate number of granules was present, the granule profile showing the largest single diameter in a section might be one cut through its long axis. Thus, measure ment of this profile might provide information as to the actual dimensions of the complete granule. One obvious limitation to such measurements would be the occasional presence of an abnormaly large granule. Observations with the light microscope suggest that this would be most likely to occur in the myelocytes where some variation in granule size has been reported rather than in the later forms where the granules are said to be more uniform (10) With

reference to this, it is noteworthy that the average values obtained for the length of the granules with interna, both for the whole series (0 9 μ) and for each developmental category are not significantly different from one another they also lie within the estimated range of normal granule length given by other authors, e g 0 7 –1.3 μ (12) and 0.5–1 0 μ (6 7) However in view of the possible limitations and errors inherent in this type of measurement, care must be exercised particularly in interpreting the smaller differences in Table II

*Granule dimensions and cell maturity* As already noted no significant differences have been detected between the three developmental categories with respect to the dimensions of the largest internum-containing profiles. In contrast, marked differences are found when the profiles of the granules which showed no evidence of an internum on section are considered As shown in Table II these profiles were much smaller in the band and segmented forms than in either the myelocytes or metamyelocytes. Such a finding does not necessarily mean that the granules without interna are smaller in size in the more mature forms it might be a consequence of their being fewer in number If this were the case the likelihood of one of them being cut through its centre by a random section would be much reduced. Furthermore some of these smaller profiles in the more mature forms might represent the peripheral part of internum-containing granules, where the section had not passed through the internum Alternative explanations would therefore seem to fit the findings equally well Either the granules without interna are fewer in number in the more mature forms of eosinophil or they are smaller in size, or perhaps they are both fewer and smaller In functional terms, a decrease in number might be brought about by their conversion to internum containing granules, whilst a decrease in their size (perhaps accompanied by a decrease in their numbers as well) might represent regression perhaps reflecting a decrease in their functional activity In the latter case the internum-containing granules would presumably have an independent origin

*Granule shape and the presence of an internum* The point which is brought out most clearly by the measurements recorded in Table II is that the largest internum-containing profiles are usually somewhat ovoid On average their long axis measured approximately twice their transverse diameter On the other hand, the largest pro-

files of the granules without interna were more rounded, their long axis measuring on average only 1.3–1.5 times their transverse diameter As shown for example in granule Gl in Fig 1 the long axis of the internum frequently lies in the long axis of the profile and it is tempting to suggest that the elongation of the internum-containing granule is directly related to the presence of the internum. The internum is presumably in a solid form and the implication would seem to be that the shape of the granule may be influenced by mechanical factors connected with this. It has been noted (8, 19) that the internum may sometimes give the appearance of stretching the limiting membrane. With regard to the granules without interna one cannot exclude the possibility that some of these represent lysosomes or other inclusions, although there is evidence that the granules of mature eosinophil leucocytes should themselves be regarded as having lysosome like functions (1) One of the possibilities mentioned in the preceding section is that at least some of the granules without interna may be converted into internum-containing granules. This would raise problems as to the method by which such a conversion might occur One possible hypothesis would be that the material of which the internum is composed is already present in a dispersed (presumably fluid) form in some of the granules without interna. The possible nature of this material has been discussed elsewhere (2, 11 17) One can imagine that as development of the granule proceeds, a critical concentration of this material might be reached, after which the formation of the internum could take place by a process of solidification On this hypothesis, the formation of the internum could be responsible for bringing about a corresponding change in the shape of the granule. One is also tempted to enquire whether the formation of the internum might not be a postmortem event. If this were the case, it would seem unlikely that it would be dependent solely on electron microscopic techniques. Ovoid eosinophil granules have been noted in ordinary haematological preparations (10) and under phase contrast (5) Studies of the chemical properties of the Charcot Leyden crystal (14) suggest by analogy that factors such as changes in hydrogen ion concentration of the internal medium of the eosinophil granule might be important in precipitating the formation of an internum in such circumstances.

In summary one may say that the evidence does not seem to permit us to go beyond the point of speculation as to the possible

mechanisms by which the internum is developed the possibility that internum-containing granules are formed independently of the other granules cannot at present be excluded Further investigation of the mechanisms suggested above would certainly appear to be merited.

*Eosinophil granules and foreign protein stimulation.* The internum-containing granule profiles were, on average, somewhat more elongated in one diameter in the eosinophils of the animals which had received repeated injections of foreign protein than in the normal This is shown by a somewhat greater profile length (and length breadth) in Table II The differences are significant at the 0 05 level but not at the 0 01 level of probability In view of the possible limitations of the method (discussed above) such differences should probably be regarded as no more than suggestive, indicating that further measurements on a larger series might be worth while.

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*Summary*

Data has been obtained relating to the granules of three development categories of eosinophil leucocyte as seen in electron micrographs of guinea-pig bone marrow Granule counts indicated that the more mature the cell, the greater was the proportion of granules showing an internum in any particular section. Measurements of the dimensions of the largest granule-profiles also suggested that the granules without interna may be fewer or smaller (or both) in the more mature forms of cell. They further indicated that the largest profiles of internum-containing granules were on average more ovoid than those of the granules showing no internum. The possible functional significance of these findings has been discussed.

*Zusammenfassung*

An drei Entwicklungsstadien eosinophiler Leukocyten aus dem Knochenmark von Meerschweinchen wurden die Granula elektronenmikroskopisch untersucht. Je reifer eine Zelle ist, desto grösser ist pro Schnitt der Anteil ihrer Granula mit einer Innenstruktur Granula ohne Innenstruktur sind spärlicher und/oder kleiner in den reiferen Zellen. Granula mit Innenstruktur weisen ein ovaleres Profil auf als solche ohne Innenstruktur Die mögliche funktionelle Bedeutung dieser Befunde wird diskutiert.

*Résumé*

Les granulations éosinophiles des leucocytes de la moelle osseuse du cobaye ont été étudiées au microscope électronique à trois différents stades de leur évolution. Plus la cellule est mature, plus le nombre des granulations ayant une structure intérieure est grand. Les granulations sans structure intérieure sont moins nombreuses ou plus petites (ou les deux) dans les cellules plus matures. Les granulations ayant une structure intérieure présentent sur coupe un profil plus oval que celles sans structure intérieure. La signification fonctionnelle possible de ces observations est discutée.

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# A Peculiar Benzidine Positive Protein Fraction in the Plasma of Newborns and Children with Various Haemolytic Conditions*

E. Bottini, G Filippi, F Cozzi and G Maggioni

The presence of a fast haemoglobin fraction in the plasma and urines of adults with Haemolytic Anaemias has recently been reported (1) In the course of our studies (2, 3 4) concerning the behaviour of some protein fractions [haptoglobin (Hp) methaemalbumin (MetAlb) free haemoglobin (Hb)] in the plasma of children in various physiologic and pathologic conditions, by means of starch-gel electrophoresis, we had observed in several instances the presence of a particular benzidine-positive band which did not correspond to the above mentioned fractions. The band appears to be identical to the fast haemoglobin fraction recently found in adults, so it seems worthwhile to report the relevant data on these investigations together with some additional observations made on the subject.

## *Methods*

Capillary blood was collected in heparinized tubes The plasma was immediately separated by centrifugation and refrigerated at +4 C

The analysis was carried out within 24–48 h by starch-gel electrophoresis according to Poulik (5) and only in few cases by the method of Smithies (6)

Acetic benzidine was used for staining

Plasma ultrafiltration was performed by Membranfilter Göttingen

## *Results*

(1) *The fast fraction* Fig 1 shows the starch-gel electrophoretic pattern obtained with plasma from an one day old premature infant. The fast band, which we shall at present refer to as 'V' (veloce) is in a more advanced position toward the positive pole as

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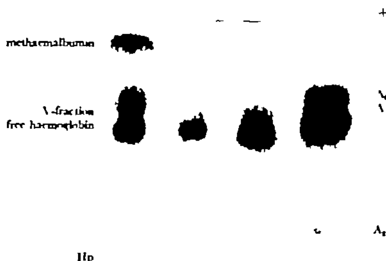



Fig 1 Plasma from one day old premature infant. Starch gel electrophoresis in discontinuous system, stained with benzidine
(1) plasma of the premature before exchange-transfusion
(2) plasma of the premature after exchange-transfusion
(3) haemolysate from the same premature
(4) haemolysate from normal adult aged 8 days

compared to the free Hb band and is closer to the latter when compared to the MetAlb band.

(2) *Relationship between the presence of the fast fraction and other fractions and its frequence in various conditions* The rate of occurrence of the V band is reported in the table on the basis of 378 plasma samples examined. The presence of the V band appeared closely related to than of free Hb (Table, columns 5 and 6)

Table also lists the relationship between the V band and the Hp band (columns 7 8, 11 12) and the MetAlb band (columns 9 10 13 14) in the cases in which free Hb was present. Considering all the cases examined, the V band was found more frequently in those without Hp the difference however was not statistically significant (0 5 >P >0 3) The presence of the V band is almost constantly associated with that of the MetAlb band when the V band is absent, the MetAlb band is prevalently absent too. The difference is statistically significant ($\chi^2$ = 48 P<0 01)

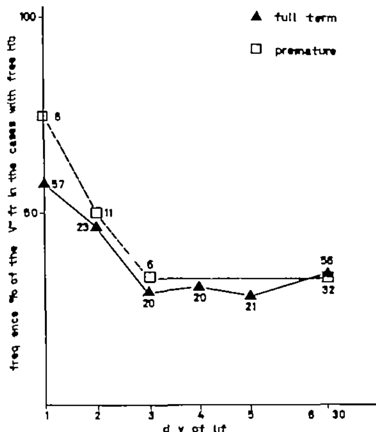



*Fig 2.* Frequence of the fast fraction in the cases with free plasma haemoglobin during the first month of life The small number indicates the total number of cases examined.

Both in full term and in premature newborns presenting free plasma Hb the V' fraction appeared more frequently in the first few days of life only to diminish gradually in the following days (Fig 2) It was found more frequently in premature than in full term infants in the first five days of life the difference however is not statistically significant ($0.5 > P > 0.3$)

We examined groups of full-term and premature infants with neonatal jaundice, with and without blood group incompatibility and searched for the V' band before any exchange transfusion was undertaken The V' band (always referred to the cases with free Hb) was present to a greater extent in infants with neonatal jaundice as compared to non jaundiced newborns it was present more fre

*Table*

Relationships between the presence of X fraction and other fractions

| Age of life days | No. of cases | Hp (1) | Met-Hb (2) | free Hb (3) | [illegible] (4) | with Hb (5) | without Hb (6) | with Hp (7) | without Hp (8) | with Met-Hb (9) | without Met-Hb (10) | with Hp (11) | without Hp (12) | with Met-Hb (13) | without Met-Hb (14) | with Hp (15) | without Hp (16) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | presence of band X % | | presence of band X % | | | | In presence of Hb absence of band X % | | | | In presence of Met-Hb absence of band X % | |
| *Normal newborn* | | | | | | | | | | | | | | | | | |
| 1 | 37 | 9 | 16 | 17 | 10 | 10 | | 2 | 8 | 9 | 1 | 3 | 1 | 2 | 5 | 2 | 5 |
| 2 | 23 | 16 | 15 | 19 | 9 | 9 | | 7 | 2 | 9 | | 5 | 5 | 6 | 1 | 2 | 1 |
| 3 | 20 | 18 | 6 | 10 | 4 | 4 | | 2 | 1 | 3 | | 6 | 1 | 2 | 5 | 3 | — |
| 4 | 20 | 11 | 8 | 13 | 1 | 1 | | 3 | 1 | 3 | 1 | 5 | 4 | 3 | 1 | 2 | 1 |
| 5 | 21 | 11 | 7 | 17 | 5 | 5 | | 1 | 1 | 3 | 2 | 6 | 8 | 1 | 8 | 1 | 1 |
| 6–30 | 54 | 37 | 18 | 31 | 11 | 11 | | 6 | 5 | 11 | | 11 | 9 | 7 | 13 | 3 | 1 |
| 30–70 | 11 | 11 | 1 | 1 | 1 | 1 | | 1 | | 1 | | | | — | — | — | — |
| *Premature* | | | | | | | | | | | | | | | | | |
| 1 | 8 | | 7 | 8 | 1 | 1 | | | 6 | 6 | | | 2 | 1 | 1 | — | 1 |
| 2 | 11 | 2 | 9 | 8 | 1 | 1 | | 1 | 3 | 1 | | 1 | 3 | 2 | 2 | 1 | 1 |
| 3 | 6 | | 5 | 6 | 2 | 2 | | | 2 | 2 | | | 4 | 1 | 3 | — | 3 |
| 4 | 1 | | | 1 | | | | | | | | | 1 | — | 1 | — | — |
| 5 | 3 | 1 | | 2 | 1 | 1 | | | 1 | | 1 | | 1 | — | 1 | — | — |
| 6–30 | 32 | 21 | 6 | 18 | 6 | 6 | | 1 | 2 | 1 | 2 | 8 | 1 | 2 | 10 | 1 | 1 |
| 30–110 | 11 | 8 | | 1 | | | | | | | — | 1 | 3 | — | 1 | — | — |

*Fullterm newborns with jaundice*

without incompatibility

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 3<br>6–30 | 7<br>14 | —<br>4 | 3<br>3 | 4<br>9 | 3<br>1 | 3<br>1 | —<br>— | —<br>1 | 3<br>— | 3<br>— | —<br>1 | —<br>2 | 1<br>6 | 1<br>3 | —<br>3 | —<br>2 | 2<br>1 |

with incompatibility

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 3<br>6–30 | 13<br>8 | 3<br>— | 3<br>3 | 3<br>3 | 1<br>— | 1<br>— | —<br>— | —<br>— | 1<br>— | 1<br>— | —<br>— | 3<br>— | 1<br>3 | 1<br>2 | 3<br>1 | —<br>— | 2<br>3 |

*Premature with jaundice*

without incompatibility

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 5<br>6–30 | 13<br>8 | 4<br>2 | 12<br>2 | 7<br>3 | 3<br>— | 3<br>— | —<br>— | 1<br>— | 4<br>— | 3<br>— | —<br>— | —<br>2 | 2<br>3 | 2<br>2 | —<br>3 | 2<br>— | 3<br>2 |

with incompatibility

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 5<br>6–61 | 4<br>10 | 2<br>3 | 2<br>3 | 3<br>3 | 2<br>— | 2<br>— | —<br>— | 1<br>— | 1<br>— | 2<br>— | —<br>— | 1<br>2 | —<br>3 | —<br>3 | 1<br>2 | —<br>1 | —<br>2 |

*Thalassaemia major*

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 9 | 3 | 9 | 3 | 1 | 1 | — | 1 | — | 1 | — | 2 | 2 | 4 | 1 | 3 | 3 |

*Thalassaemia minor*

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 8 | 8 | — | 1 | — | — | — | — | — | — | — | 1 | — | — | 1 | — | — |

*Foetus*

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | — | 4 | 2 | 4 | 2 | 2 | — | 2 | 2 | — | — | — | — | — | — | — |

quently in infants without blood incompatibility than in infants with incompatibility (the differences however are not statistically significant $0.5 > P > 0.3$ and $P^* = 0.12$ respectively *)

In nine children with thalassaemia major the V' band was present only once out of five with free Hb whereas in the parents of these patients (eight cases examined) the V' band was constantly absent.

In four cases of favism with haemolytic crises, the V' band was constantly present up to the 5th or 6th day following the crises. In few cases in which the analysis of urine was performed it was found a benzidine positive fraction in a position intermediate between free Hb and MetAlb.

We have observed that the ageing of plasma containing free Hb is accompanied by an increase of the V' band however this fraction was present also in samples tested immediately after withdrawal whereas in other samples it did not appear even after few days of ageing

(3) *Preliminary experiments in the identification of the V' band* 0.5 / and 0.3 / solutions of Hb (obtained from cord blood haemolysates) were incubated at 37 C for approximately 12 h with plasma obtained from a one day old premature infant (0 6 kg) from the umbilical cord of a full-term infant and from a normal adult separately The solutions were then examined by means of starch-gel electrophoresis. The V' band was present in the first case it was less apparent in the second case, whereas solutions containing adult plasma yielded no V' band at 0.3 / and only a slight trace at 0.5 / concentration. MetAlb was present constantly Haemolysates without plasma did not present any band comparable to the V band

When the experiment was repeated with the ultrafiltrate of the above mentioned plasmas, the band did not appear but it was again observed when a concentrate of the plasmas was used (Fig 3)

Similar results were obtained by incubating normal adult Hb with umbilical cord plasma. We also incubated the same cord plasma with canine Hb the latter is known to have a lesser electrophoretic mobility (at an alkaline pH) than Hb A. Electrophoresis revealed besides a MetAlb band a benzidine positive band whose advanced position in respect to the canine band was comparable to the advanced position of the V' band in respect to the Hb A

$P^*$ = Probability calculated according to Fisher.




*Fig. 3.* Human Hb incubated for 12 h at 37 °C with concentrate and ultrafiltrate from plasma of normal adult, premature and full-term newborn. Starch gel electrophoresis in discontinuous system, staining with benzidine.
*1* Hb. *2* Adult plasma concentrate + Hb. *3* Adult plasma ultrafiltrate + Hb. *4* Premature plasma concentrate + Hb. *5* Premature plasma ultrafiltrate + Hb. *6* Full-term newborn plasma concentrate + Hb. *7* Full-term newborn plasma ultrafiltrate + Hb.

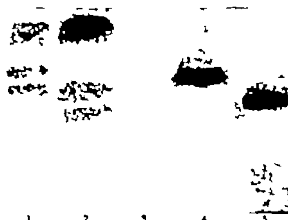



*Fig. 4.* Canine and human Hb incubated with umbilical cord plasma. Starch gel electrophoresis in discontinuous system. Staining with benzidine.
*1* Umbilical cord plasma + human Hb. *2* Umbilical cord plasma + canine Hb. *3* Umbilical cord plasma. *4* Human Hb. *5* Canine Hb.

band (Fig. 4). Electrophoretic analysis of plasmas with Hp type 1/1 and 2/1 mixed with canine Hb showed that the mobility of the canine Hb/Hp complex was identical to that of the Hp and Hb A complexes.

Further experiments were carried out by using different cord plasmas and the same results were obtained however the intensity of the band seemed to vary with each plasma. Furthermore after preserving the plasma for approximately fifty days at 4 C, the result was less apparent. Also the incubation of canine plasma with human or canine Hb exceeding its Hb binding capacity yielded a benzidine positive band in a more advanced position

*Discussion*

The V" band could represent a globulin-haematin complex its position corresponds roughly to that described for haemopexin (7) The latter is a $\beta_1$B globulin (8) capable of binding haematin but probably not Hb.

Hypothetically the V band may represent a product of transformation of Hb in fact its position corresponds to that of Hb $A_2$. This would derive from Hb A through the ageing of the red cell and its quantity increases with the age of the sample (9 10) HUISMAN (11) obtained a Hb with characteristics similar to those of Hb $A_2$ by incubating Hb A with oxidized glutathione in this case the glutathione is bound to the $\beta$-chain and the analysis following tryptic digestion reveals an additional spot other than the Hb A fingerprint corresponding to GSH Presence of GSH in the Hb $A_2$ fingerprint has been observed (12) However HUISMAN believes that Hb $A_2$ obtained from fresh samples differs from Hb obtained by incubation with oxidized glutathione The nature of Hb $A_2$, as found *in vivo* is not clear and one cannot exclude the possibility of a formation of complexes of Hb A with other substances. Further more one may advance the hypothesis that complexes of Hb with oxidized glutathione are formed in particular conditions in which reduction of the latter is lacking

*The relationship between V" fraction and MetAlb* may be explained by both these hypotheses. The constant finding of free Hb in the presence of band V" and its increasing intensity with the ageing of sample favor the theory that band V" is a product of transformation of Hb

The results of our preliminary experiments *in vitro* seem to confirm that band V" is to a large extent a result of the transformation of Hb and that in plasma there are non-ultrafiltrable factors capable of accelerating Hb transformation as compared to haemo-

lysates. These factors seem to be more active in the first few day of life especially in the premature infants however foetal red blood cell factors and absence of Hp may be important in increasing the frequence of \ fraction in this period. In all probability also the concentration of Hb plays a part in the formation of the fast fraction.


## Summary

In the plasma of newborn infants and of children with arious haemolytic diseases, benzidine positiv protein fraction has been observed tha shows an electrophoretic mobility greater than free haemoglobin and lesser than methaemalbumin. The analysis of plasmas and experiments *in vitro* seem t support the hypothesis that the fraction represents mainly derivative of Hb (type Hb $A_4$) and that the plasma contains non-ultrafiltrable substances capable of increasing the rat of transformation from the normal to the fast type, as compared with that observed in the haemolysates. I seems that the abov substances are more activ during the first few days of life especially in the premature.

## Zusammenfassung

Im Plasma von Neugeborenen und Kindern mit erschiedenen hämolytischen Krankheiten wurde eine besondere Benzidin-positive Proteinfraktion beobachtet, die bei der Elektrophorese eine großere Geschwindigkeit als das freie Hamoglobin aufweist.

Untersuchungen *in vitro* stutzen die Annahme, daß diese Fraktion, wenigstens zum großen Teil, aus einem Umwandlungsprodukt des Hb ( om Typ $HbA_4$) besteht und daß im Plasma nicht-ultrafiltrierbare Faktoren orkommen, die die Verwandlung rascher als beim Hämolysierten Praparat beeinflussen. Es scheint, daß diese Faktoren in den ersten Lebenstagen aktiver sind, besonders bei F uhgeburten.

## Résumé

Dans le plasma d'enfants nouveau-nes ueurts de differents maladies hémolytiques une fraction protéinique, benzidine-positi été observée qu une mobilité électrophorétique plus grande que l'hémoglobine libre et plus faible que la methemalbumine. Les résultats d'expériences in itro semblent étayer l'hypothèse selon laquelle cette fraction représenterait principalement un dérivé de l'hémoglobine (type $HbA_4$) et que le plasma contiendrait des substances non-ultrafiltrables capables d' ugmenter par rapport aux hémolysats la transformation de type normal en type rapide. Il semble que ces substances soient plus actives duran les premiers jours de la vie surtout chez les nouveau-nés prématurés


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# Über den Retraktionsvorgang beim Blutplasmagerinnsel

A. Fonio

Neuere Untersuchungen mit dem Dunkelfeldverfahren haben ergeben, daß am Aufbau des Blutplasmagerinnsels zwei Grundsubstanzen beteiligt sind

1 Das dem Fibrinogen entstammende Fibrin weist anfangs eine Andeutung von Gerinnungsstreifen auf (Abb 1) erscheint in der Folge jedoch als völlig strukturlose, und in dünner Schicht im Deckglaspräparat, als dichte nebelartig aufgehellte Masse mit einzelnen, kleinen, runden, hellen Flecken (Abb 2)

2. Die das Gerinnsel durchziehenden Fibrillen entstehen aus Proteintropfen oder massen (Protein I) und liegen entweder frei auf der Fibrinmasse, davon leicht uberdeckt oder dann völlig eingeschlossen. Einzelne Fibrillen können aus der Fibrinmasse in den freien Raum austreten (Abb 19 20 21 22, 23)

Die Fibrillen weisen charakteristische Strukturen auf Sie sind *a)* fadenförmig und einerseits aus reihenförmig hintereinander anklebenden, spitzendigenden Proteintropfen auf dem strukturlosen Fibringerinnsel gelagert (Abb 3 4) oder *b)* fibrillenförmig und andererseits auf oder im ausgefallenen Fibringerinnsel durchziehend Die Fibrille besteht aus zwei parallel zueinander stehenden Langsrand rn, die einen Langsleerraum umschließen, der die ganze Länge der Fibrille durchzieht (Abb 5 16) Dieser Aufbau des Fibringerinnsels und der einzelnen Fibrillen findet sich sowohl beim thrombozytenhaltigen als beim thrombozytenfreien Plasma vor Der Retraktionsvorgang der nur im thrombozytenhaltigen Plasma, bzw Gerinnsel stattfindet, beginnt am Proteingehalt II im Fibrillenlängsleerraum zunachst mit den Erscheinungen der Querstreifung (Abb. 6) sodann mit dem Ausscheiden in quere kontraktile und nichtkontraktile Schichten bei Erhaltung des Fadenquerschnittes Abb 7) weiter mit Übergreifen derselben auf die Fibrillenränder und daruber hinaus auf das Fibringerüst und so zur

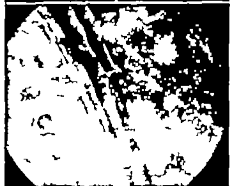
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1


2


3


4


5


6


7


8

Retraktion des Gesamtgerinnsels durch die an den Fibrillen anklebenden Fibrinmassen führend (Abb 8)

Die einzelnen Fibrillen können sich, ähnlich wie die Myofibrillen zur Muskelfaser zu Fibrillensträngen zusammenschließen Beiden kommt eine verkürzende Wirkung zu den Myofibrillen eine direkte die Kontraktion der Muskelfaser den Fibrillen, einzeln oder als Fibrillenstränge eine indirekte, fibrinzusammenraffende zur Retraktion des Gesamtgerinnsels führend.

Das ausgefallene in dünner Schicht im Deckglaspräparat befindliche Fibrin verhält sich wie eine plastische, leicht umzuformende, an den Fibrillen adhärierende Substanz, die so beim Retraktionsvorgang mitgerissen wird, was beim Austritt einer Fibrille aus dem Gerinnsel in den Leerraum gut ersichtlich ist (Abb 8)

Als dritte Grundsubstanz beim Retraktionsvorgang kommt beim thrombozytenhaltigen Plasma zusätzlich der Proteingehalt II im Längsquerraum der Fibrillen mit den charakteristischen Querstreifungsbefunden in Betracht (Abb 6, 7) Im folgenden wird das *Verhalten der Grundsubstanzen beim thrombozytenfreien Plasma* erörtert.

## *Untersuchungsmethoden*

*I Thrombozytenhaltiges Plasma*

1 9 ml Blut + 1,0 ml Natr. citric. 2,5%
2. Zentrifugierung der Mischung 3 min lang bei 1500 U /min,
3. abheben der thrombozytenhaltigen Plasmaschicht,
4. rekalzifieren Zu 0,5 ml Plasma 1 Tropfen $CaCl_2$ 2%

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*Abb. 1* Andeutung von Gerinnungsstreifen im frisch ausgefallenen Fibrin.

*Abb. 2.* In der Folge strukturlosen Fibrin mit winzig kleinen, runden, hellen Flecken in der Fibrinmasse verteilt (Protein I)

*Abb. 3.* Fadenförmige Fibrille aus aneinander gereihten und verklebten, spitzendigenden Proteintropfen I auf strukturloser Fibrinmasse gelagert.

*Abb. 4* Fadenförmige Fibrille aus aneinander und nebeneinander gelagerten Proteintropfen I spitzendigend, auf strukturlosem Fibringerinnsel gelagert.

*Abb. 5.* Fertig gebildete Fibrille aus zwei parallel zueinander gelagerten Längsrändern, einen Längsleerraum durch die ganze Fibrillenlänge hindurch bis zu deren Ende umschließend.

*Abb. 6.* Querstreifungsschichten im Proteingehalt II des Längsleerraumes beim Retraktionsvorgang des thrombozytenhaltigen Gerinnsels.

*Abb. 7* Kontraktile und nicht kontraktile Schichten im Proteingehalt II des Längsleerraumes des thrombozytenhaltigen Gerinnsels beim Retraktionsvorgang

*Abb. 8.* Austritt einer Fibrille aus dem thrombozytenhaltigen Fibringerinnsel in den Leerraum, adhärierendes Fibrin mitreißend.

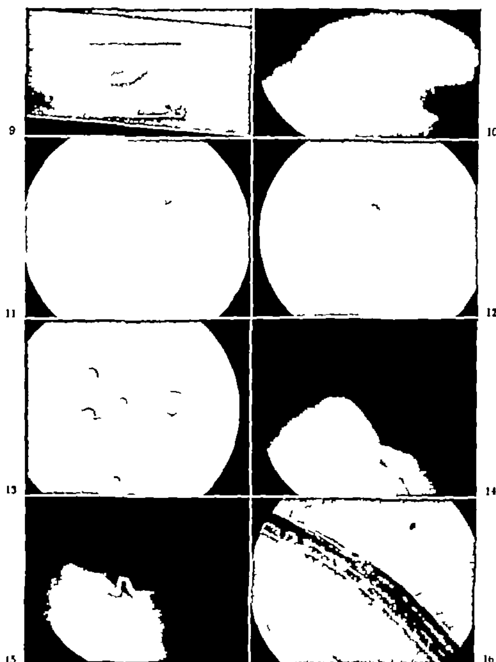



Abb. 9. Thrombozytenfreies Gerinnsel, entnommen dem Mikroretraktionsröhrchen mit den Spitzenbräuschen einer Pipette. Ein Deckglaspräparat, im Dunkelfeldmikroskop untersucht und photographiert.

5. beschicken der Mikroretraktionsröhrchen mit dem rekalzifizierten Plasma,

6. fassen der retrahierten Oberflächenschicht der Gerinnsel mit den Spitzenbranchen einer Pinzette, herausheben der Gerinnsel,

7. ablegen des Gerinnsels auf Objektträger – bedecken mit Deckglas – gute Verteilung des Serums abwarten. Verbringung in Dunkelfeldmikroskop (Abb. 9)

*II. Thrombocytenfreies Plasma*

1. 2, 3 wie bei I.,

4. Zentrifugieren der abgehobenen Plasmaschicht eine Stunde lang bei 4000 U/min: *thrombocytenfreies Plasma*,

5. rekalzifizieren: zu 0,5 ml Plasma 1 Tropfen $CaCl_2$ 2%,

6. beschicken der Mikroretraktionsröhrchen mit dem rekalzifizierten Plasma,

7. fassen der Oberflächenschicht des Gerinnsels mit den Spitzenbranchen einer Pinzette und Herausziehen des Gerinnsels,

8. ablegen des Gerinnsels auf Objektträger – Bedeckung mit Deckglas – gute Verteilung des Serums abwarten. Verbringung in Dunkelfeldmikroskop (Abb. 9)

## *Resultate*

*Verhalten der Grundsubstanzen des thrombocytenfreien Plasmas beim Gerinnungsvorgang des Fibrins und der Fibrillen.* Das ausgefallene Fibrin unterscheidet sich nur wenig von demjenigen des thrombozytenhaltigen Plasmas: dichte, nebelartig aufgehellte Masse bei erhöhter Zahl von kleinsten, runden, hellaufleuchtenden Flecken (Abb. 10) bei größerer mikroskopischer Vergrößerung sich als kugelförmig einzelnen oder aus mehreren zusammengesetzten

25 mm lang, 2 mm breit. Präparierung: mechanische Reinigung mit warmem Wasser und mit Seife, hernach mit Vel-Lösung; mit heißem Wasser nachspülen, ... mit Aqua dest.; bei Wärme trocknen lassen; vor Gebrauch mit Bunsenbrenner ausglühen. (Nur bei derart vorbereiteten Mikroretraktionsröhrchen erfolgt die Retraktion ungestört ohne Ankleben an die Glaswand.)

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*Abb. 10.* Ausgefallene Fibrinmasse, nebelartig aufgehellt, mit zahlreichen, runden, hellaufleuchtenden Flecken = Protein I. Fibrillengrundsubstanz (Fibrillinogen)

*Abb. 11.* Protein I bei starker Vergrößerung

*Abb. 12.* Protein I gehäuft bei starker Vergrößerung

*Abb. 13.* Protein I blasenförmig gehäuft bei starker Vergrößerung, darüber und darunter einzelne Fibrillenandeutungen.

*Abb. 14.* Querverlaufende, dunkle Fibrinfalten, gardinenähnlich geordnet und auf erhöhte Plastizität des Fibrinaufbaues hindeutend, vermutlich durch Seitenstoß gelinder Art entstanden.

*Abb. 15.* Schwanzähnlich aus dem *Gerinnsel* austretende wellenförmige *Fibrille*, von plastischem Fibrin total überdeckt.

*Abb. 16.* Struktur einer auf strukturloser Fibrinmasse gelagerten Fibrille aus thrombozytenfreiem Plasma, mit den zwei Längsrändern und mit eingefaßtem durchgehenden Längsleerraum.

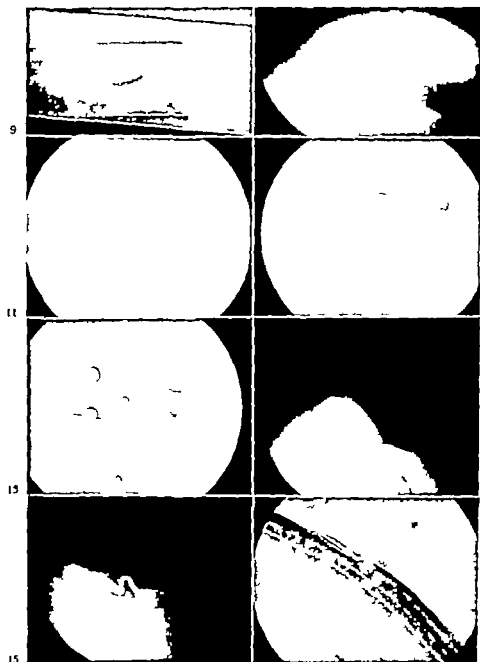



Abb. 9. Thrombocytenfreies Gerinnsel, entnommen dem Mikroretraktionsröhrchen mit den Spitzenbranchen einer Pinzette. Ein Deckglaspräparat, im Dunkelfeldmikroskop untersucht und photographiert.

5. beschicken der Mikroretraktionsröhrchen mit dem rekalzifierten Plasma,

6. fassen der retrahierten Oberflächenschicht der Gerinnsel mit den Spitzenbranchen einer Pinzette, herausheben der Gerinnsel,

7. ablegen des Gerinnsels auf Objektträger – bedecken mit Deckglas – gute Verteilung des Serums abwarten – Verbringung in Dunkelfeldmikroskop (Abb. 9)

*II. Thrombozytenfreies Plasma*

1, 2, 3 wie bei I.,

4. Zentrifugieren der abgehobenen Plasmaschicht eine Stunde lang bei 4000 U/min: *thrombozytenfreies Plasma*

5. rekalzifieren: zu 0,5 ml Plasma 1 Tropfen $CaCl_2$ 2%,

6. beschicken der Mikroretraktionsröhrchen mit dem rekalzifierten Plasma,

7. fassen der Oberflächenschicht des Gerinnsels mit den Spitzenbranchen einer Pinzette und Herausheben des Gerinnsels,

8. ablegen des Gerinnsels auf Objektträger – Bedeckung mit Deckglas – gute Verteilung des Serums abwarten – Verbringung in Dunkelfeldmikroskop (Abb. 9)

## *Resultate*

*Verhalten der Grundsubstanzen des thrombozytenfreien Plasmas beim Gerinnungsvorgang des Fibrins und der Fibrillen.* Das ausgefallene Fibrin unterscheidet sich nur wenig von demjenigen des thrombozytenhaltigen Plasmas: dichte, nebelartig aufgehellte Masse bei erhöhter Zahl von kleinsten, runden, hellaufleuchtenden Flecken (Abb. 10), bei größerer mikroskopischer Vergrößerung sich als kugelförmig einzelnen oder aus mehreren zusammengesetzten

25 mm lang, 2 mm breit. Präparierung: mechanische Reinigung mit warmem Wasser und mit Seife, hernach mit Vel-Lösung; mit heißem Wasser nachspülen, dl. mit Aqua dest.; bei Wärme trocknen lassen; vor Gebrauch mit Bunsenbrenner ausglühen. (Nur bei derart vorbereiteten Mikroretraktionsröhrchen erfolgt die Retraktion ungestört ohne Ankleben an die Glaswand.)

*Abb. 10.* Ausgefallene Fibrinmasse, nebelartig aufgehellt, mit zahlreichen, runden, hellaufleuchtenden Flecken = Protein I, Fibrillengrundsubstanz (Fibrillinogen)

*Abb. 11.* Protein I bei starker Vergrößerung.

*Abb. 12.* Protein I gehäuft bei starker Vergrößerung

*Abb. 13.* Protein I blasenförmig gehäuft bei starker Vergrößerung, darüber und darunter einzelne Fibrillenandeutungen.

*Abb. 14.* Querverlaufende, dunkle Fibrinfalten, gardinenähnlich geordnet und auf erhöhte Plastizität des Fibrinaufbaues hindeutend, vermutlich durch Seitenstoß gelinder Art entstanden.

*Abb. 15.* Schwanzähnlich aus dem Gerinnsel austretende wellenförmige Fibrille, von plastischem Fibrin total überdeckt.

*Abb. 16.* Struktur einer auf strukturloser Fibrinmasse gelagerten Fibrille aus thrombozytenfreiem Plasma, mit den zwei Längsrändern und ein eingefaßtem durchgehenden Längsleerraum.

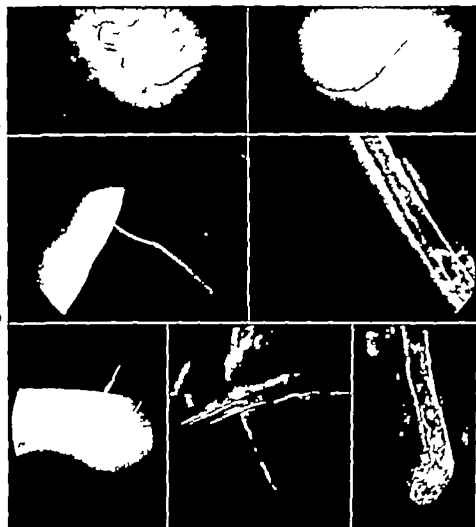



Abb. 17. Fibrille aus thrombozytenfreiem Gerinnsel, schlaff und wellig auf strukturloser zwei Tage alter Fibrinmasse von fleckigem Aussehen liegend.

Abb. 18. Fibrille wie bei Abb. 17 teilweise von Fibrin bedeckt.

Abb. 19. Aus dem Gerinnsel in den Leerraum austretende Fibrille.

Abb. 20. Gleiche Fibrille wie Abb. 19 bei starker Vergrößerung, bei leerem Längsraum und ohne Retraktionsandeutung.

Abb. 21. In den Leerraum ausgetretene geknotete Fibrille bei thrombozytenfreiem Gerinnsel.

Abb. 22. Gleiche Fibrille wie Abb. 21 bei starker Vergrößerung, sich überkreuzende Fibrillen mit leerem Längsleerraum.

Abb. 23. Ausgetretene Fibrille von thrombozytenfreiem Gerinnsel, mit leerem Längsleerraum bei abgerundetem Ende.

Proteingebilden oder Proteinblasen erweisend (Protein I) (Abb. 11, 12, 13). Nach Erscheinen der neugebildeten Fibrillen im ausgefallenen Fibrin finden sich nur noch seltenere helle, runde Flecken, so daß angenommen werden kann, daß der Großteil der erwähnten Proteingebilde zur Bildung der Fibrillen aufgebraucht wurde.

Nach erfolgter Fibrillenbildung weist die dünne Fibrinschicht im Deckglaspräparat zuweilen quer- oder längslaufende dunkle, lange Falten auf, ähnlich solcher einer hohen Fenstergardine, vermutlich durch gelinden, passiven Seitendruck oder -stoß entstanden (Abb. 14), was auf eine erhöhte Plastizität des Fibrins in dünner Schicht unter dem Deckglas schließen läßt. So erklärt sich das Bild eines aus dem Gerinnsel austretenden, aufgerollten »Fibrinschwanzes« durch die Fibrinumhüllung einer aus dem Gerinnsel austretenden wellenförmigen Fibrille (Abb. 15).

Die Struktur der Fibrillen des thrombozytenfreien Plasmas entspricht vollauf derjenigen des thrombozytenhaltigen: zwei zueinander parallel verlaufende Längsränder einen durch die ganze Fibrille verlaufenden leeren Längsleerraum umschließend (Abb. 16).

*Vergleich zwischen dem Verhalten der Fibrillen des thrombozytenhaltigen Gerinnsels beim Einsetzen des Retraktionsvorganges und dem der Fibrillen des keine Retraktion aufweisenden thrombozytenfreien Gerinnsels.* Bei beiden Gerinnseln ist die Lage der Fibrillen zur Fibrinmasse identisch. Beide können das ausgefallene Fibrin *in toto* durchziehen, und oberflächlich gelegen (Abb. 17) oder leicht von Fibrin überdeckt (Abb. 18) oder sind tief darin steckend, ähnlich den Eisendrähten eines armierten Betons. Bei beiden Gerinnseln können längere Anteile der Fibrillen aus dem Gerinnsel in den Leerraum austreten (Abb. 19, 20, 21, 22, 23). Die Fibrillen des thrombozytenhaltigen Gerinnsels adhärieren dabei vielfach fest an den Glasgefäßwänden, sie ziehen, sich beim Retraktionsvorgang verkürzend, die adhärierenden Fibrinmassen *mit zur vollendeten Retraktion* des Gerinnsels heran (Abb. 8). Demgegenüber bleiben die Fibrillen des thrombozytenfreien Gerinnsels nach erfolgter Gerinnung passiv, ohne Adhaesionstendenz bei schlaffem Gerinnsel (Abb. 19, 21).

Der Unterschied dieses Verhaltens zwischen beiden Fibrillenarten bei der Herausnahme der Gerinnsel aus den Mikroretraktionsröhrchen mit den spitzen Branchen einer Pinzette zeigt ebenfalls, wie ganze Bündel langer Fibrillen und Fibrillenstränge dabei aus den Fibrinmassen herausgerissen und so freigelegt werden (Abb. 24, 26, 28, 30).

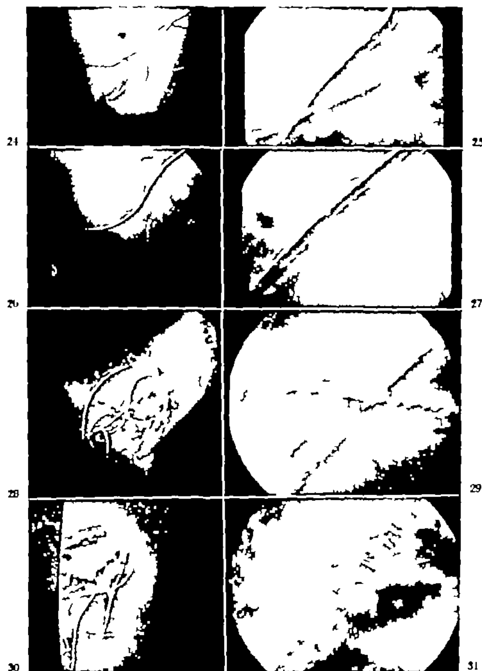



Abb. [illegible] Fibrillenbündel aus thrombocytenfreiem Gerinnsel. Fibrillen schlaff, wellenförmig, sich mehrfach überkreuzend.

Bei den Fibrillenbündeln der thrombozytenfreien Gerinnsel sind die einzelnen geraden und die sich überkreuzenden Fibrillen glatt und wellenförmig (Abb 24 26) und weisen bei starker Vergrößerung (1080 ×) die charakteristischen, parallel zueinander stehenden Längsränder mit dazwischen gefaßtem völlig leerem Längsleerraum auf (Abb 25 27)

Die Fibrillen der thrombozytenhaltigen Gerinnsel sind demgegenüber bei beginnendem Retraktionsvorgang quergestreift, steifer und länger abgerundet (Abb 28 30) und weisen bei starker Vergrößerung in dem von den zwei Längsrändern eingefaßten Längsleerraum zu Beginn des Retraktionsvorganges breite Bänder mit dazwischen durchgehenden Z Linien (Abb. 29 31) und nach erfolgter Retraktion kontraktile und nichtkontraktile einander abwechselnde Schichten bei Erhaltung des Fadenquerschnittes auf (Fig 32) Nach einiger Zeit erscheint sodann nach vorausgegangener Aufquellung und Auflösung des Inhalts Abb 33) der Längsleerraum völlig entleert. Nach einigen Tagen lösen sich die unter Deckglas gehaltenen Fibrillen unter Arrosionserscheinungen ihrer Ränder auf.

*Diskussion*

Der Vergleich zwischen den Endprodukten des *Gerinnungsvorganges* des thrombozytenhaltigen und denjenigen des thrombozytenfreien Blutplasmas ergibt, daß sie identisch und von gleicher Struktur sind sowie aus den gleichen Grundsubstanzen entstehen

I Das dem Fibrinogen ausfallende Fibrin stellt beim Aufbau des Gerinnsels eine nebelartige dichte aufgehellte Masse dar (Abb. 2, 10)

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*Abb* 25. Gleicher Fibrillenbündel bei starker Vergrößerung Längsleerräume leer glatt ohne Andeutung von Querstreifung und kontraktilen Schichten

*Abb* 26. Fibrillenbündel im thrombozytenfreien Gerinnsel, zum Teil freiliegend, zum Teil von Fibrin leicht bedeckt, wellig und schlaff

*Abb* 27 Fibrille aus Bündel wie Abb 26, mit glatten Längsrändern und leerem Längsleerraum bei starker Vergrößerung

*Abb.* 28. Fibrillenbündel aus thrombozytenhaltigem Gerinnsel. Fibrillen steif, bogenförmig verlaufend, sich überkreuzend, quergestreift Retraktionsbeginn

*Abb* 29. Gleiches Bündel wie bei Abb 28 bei starker Vergrößerung, sich überkreuzend mit kontraktilen Schichten

*Abb* 30 Fibrillenbündel wie bei Abb 28 mit Querstreifung der Fibrillen

*Abb* 31 Fibrille aus Fibrinbündel wie Abb 30 Kontraktile und nicht kontraktile Schichten im Längsleerraum breite Bänder mit durchgehenden Z-Linien.

periment nach WERNER KUHN ergibt manche Analogie bei beiden wird die Verwandlung von chemischer in mechanische Energie durch Zusatz eines Mediums zur Einbettungsflüssigkeit erreicht beim teino-chemischen verkürzt sich die Lamelle bei Zusatz von Salzsäure zur Einbettungsflüssigkeit, hebt daraufhin ein Gewicht in die Höhe (Abb. 34c) und verrichtet demnach mechanische Arbeit.

Beim physiologischen scheinbar spontan entstehenden Retraktionsvorgang reihen sich im Längsleerraum der Fibrille nach einem Übergang von breiten scharf voneinander getrennten, gleichbreiten und rechteckigen Bändern (Abb 6 31) runde münzenförmige auseinanderliegende und miteinander fest verknüpfte kontraktile und nichtkontraktile Schichten (Abb 7 32) und gewährleisten durch ihre Retraktion bei Erhaltung des gleichmäßigen Querschnittes die Kontinuität des Gerinnsels und den Schutz gegen physikalische traumatische und andersartige Verletzbarkeiten, derart eine dauernde mechanische Arbeit leistend.

Die Umwandlung von chemischer in mechanische Energie wird offenbar auch beim physiologischen Vorgang der Retraktion wie beim teino-chemischen Versuch durch die Einwirkung einer Säure beim Kuhnschen Experiment, und offensichtlich bei der physiologischen Retraktion durch die bei den glykolytischen in den Thrombozyten bei der Retraktion offensichtlich vorgehenden Prozessen ausgelöste Milchsäure

## *Zusammenfassung*

Am Gerinnungsvorgang sowohl des thrombozytenhaltigen als des thrombozytenfreien Blutplasmas beteiligen sich zwei Grundsubstanzen das Fibrinogen zum Ausfall des Fibrins und das Protein I zur Bildung der die Fibrinmasse durchziehenden Fibrillen. Die Struktur der Fibrillen ist bei beiden Plasmata charakteristisch zwei parallele Längsränder einen Längsleerraum umschließend, der die ganze Fibrille durchzieht. Der Retraktionsvorgang beim thrombozytenhaltigen Gerinnsel geht vom Protein II im Längsleerraum der Fibrillen aus Querstreifung, Umscheidung in kontraktile und nicht kontraktile Schichten bei konstantem Querschnitt, Übergreifen auf die Längsränder auf die Fibrinmasse und Retraktion des Gesamtgerinnsels Der Längsquerraum der Fibrillen des thrombozytenfreien Gerinnsels bleibt leer ohne Andeutung eines Retraktionsvorganges.

## *Summary*

Two basic substances are involved in coagulation of both thrombocyte-containing and thrombocyte-free plasma fibrinogen for the precipitation of fibrin and protein I for the formation of the fibrils which permeate the fibrin mass. The structure of the

fibrils is characteristic in both sorts of plasma—two parallel long sides enclosing longitudinal space, which occupies the whole length of the fibril. The process of retraction in plasma containing thrombocytes starts with protein II in the longitudinal space of the fibrils transverse striations, separation into contractile and non-contractile layers while the transverse diameter remains constant, encroachment on the long sides, on the fibrin mass and then traction of th whole clot. The longitudinal space of the fibrils in thrombocyte-free plasma clot remains empty of any appearance of retraction process.

## *Résumé*

Deux substances de base participent au processus de coagulation du plasma sanguin, soit qu'il contiennent des thrombocytes, soit qu il en contiennent pas le fibrinogène se transforme par précipitation en fibrine, la protéine I forme les fibrilles traversant les masses de fibrine. La structure des fibrilles est caractéristique dans les deux sortes de plasma deux bords longitudinaux renferment un espace vide longitudinal dans toute la longueur des fibrilles. Le processus de rétraction du caillot commence dans l'espace vide longitudinal des fibrilles formation de rayures, séparation en bandes contractiles et non-contractiles, le diamètre restant le même, puis le processus étend aux bords longitudinaux et à la masse de fibrine, rétraction de tout le caillot. L espace vide longitudinal des fibrilles du caillot ne contenant pas de thrombocytes reste vide et ne présente aucun des signes du processus de rétraction.

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lymphocytes (the average percentage within the group of all blood mononuclears about 2%) medium-sized lymphocytes (12%) small lymphocytes (80%) large basophilic cells (1%) lympho-monocytes (2%) and monocytes (3%) In addition the group of small lymphocytes was subdivided into 1) lymphocytes with a pale cytoplasma (about 60 to 70%) and those with basophilic cytoplasma (30 to 40%) (22) and 2) lymphocytes with nucleoles (80 to 90%) and those without nucleoles (10 to 20%) (2) In each skin window cover slip and in each corresponding blood smear we differentiated more than 1000 mononuclear cells, at least 100 of each cell type. The percentage of labelled cells and the grain count per a labelled cell (silver grains in the autoradiography of the thymidine-labelled preparations and carbon grains in the ink labelled preparations) were determined for each cell type.

## *Results*

(1) *Nuclear labelling with $H^3$-thymidine* 14 to 26 hours after the last injection of $H^3$ thymidine i e. corresponding to the 12 and 24 hour stages of the skin window No. I we found an average labelling as listed in Table I Labelling was greatest in the large basophilic mononuclear cells of the blood, almost the same in the blood monocytes, a little less in the large lymphocytes, clearly less in the medium sized lymphocytes and low in the small lymphocytes of the blood. Because of the great amount of the weakly labelled small lymphocytes the average labelling of all mononuclear cells of the blood is relatively low The average labelling of the large mononuclear cells of the blood (large basophilic cells, large lymphocytes, lympho-monocytes and monocytes) is rather high. Within the group of small lymphocytes the basophilic forms had a somewhat higher labelling index (about 20 / labelled) than the cells with pale cytoplasma (about 12 / labelled) The labelling of the small lymphocytes with nucleoles did not differ from those without nucleoles. Nearly all skin window mononuclears are heavily labelled (Fig 1 and 2) The large cells which are the most frequent cell type of the skin window mononuclears present a somewhat higher label than the medium-sized and small skin window cells.

The labelling pattern of the skin window cells with round nucleus (about 20 /) and of those with indented nucleus (80 /) is the same. Whereas the mean grain count per labelled cell group in the blood and in the skin window are relatively similar there are great differences in the percentages of labelled mononuclears between the blood and the skin window Only the large mononuclear cells of the skin window and the blood monocytes show a close correspondence such as the granulocytes of the blood and those of the skin window The medium-sized and small lymphocytes of the blood have a quite different labelling than the skin window

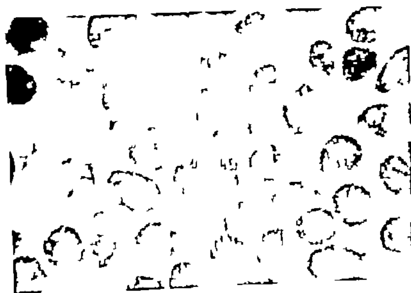


*Fig 1* Skin window No. I (24th h of inflamma n F last injection of H³-thymidine) Closely packed pr dominantly med n ndow mono-nuclears. Nearly all cells are labelled Kodak AR 10 pp film Giemsa stain 1150)

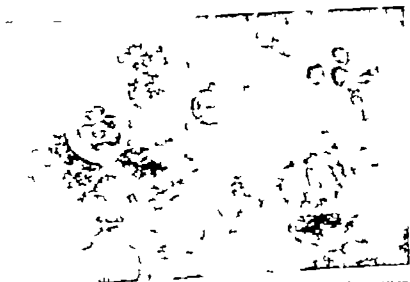


*Fig 2* Skin window No. I (26th hour of inflammation, 28 h after the last injection of H³-thymidine) All large mononuclear skin window cells macrophages) are labelled. Kodak AR 10 stripping film. Giemsa stain ( 1150)

cells including the medium-sized and small inflammatory cells. Eight days after the last injection of $H^3$ thymidine the labelling of blood and skin window cells (skin window No II) had markedly changed as shown in Table II The large basophilic mononuclear cells and the monocytes of the blood had lost their label almost completely just as the skin window mononuclears had done The large and medium sized lymphocytes and lympho-monocytes such as the average of all mononuclears and of the large mononuclears of the blood had still a little but distinct labelling whereas the small lymphocytes (with exception of the basophilic forms, which showed a lower labelling index) were similarly labelled as during the skin window I Here can only be found a parallelism between the monocytes or the large basophilic mononuclears of the blood and the skin window mononuclears. All other blood mononuclears show quite a different labelling especially the small lymphocytes. The granulocytes of the blood and those of the skin window show a close correspondence again.

(2) *Cytoplasmic labelling with India-ink* 36–48 h after the last injection of India ink—corresponding to the 12–24 hour stages of

*Table I*

Percentage of labelled cells and mean grain count per labelled cell 14 to 26 h after period of labelling with $H^3$-thymidine

| Blood | | | Skin window I | | |
|---|---|---|---|---|---|
| Cell type | Percent of labelled cells | Grain count per labelled cell | Percent of labelled cells | Grain count per labelled cell | Cell type |
| All mononuclear cells | 32.1 | 16.0 | 81.4 | 16.8 | all mononuclear cells |
| Large mononuclear cells | 75.5 | 16.9 | 85.2 | 17.5 | large mononuclear cells |
| Large lymphocytes | 62.5 | 17.5 | | | |
| Medium-sized lymphocytes | 46.7 | 17.3 | 74.0 | 16.3 | medium-sized mononucl. cells |
| Small lymphocytes | 15.3 | 13.6 | 70.0 | 13.1 | small mononuclear cells |
| Large basophilic mononuclear cells | 96.0 | 22.3 | | | |
| Lympho-monocytes | 70.2 | 15.0 | | | |
| Monocytes | 90.7 | 17.2 | 85.2 | 17.5 | large mononuclear cells (macrophages) |
| Granulocytes | 87.2 | 8.0 | 84.8 | 9.2 | granulocytes |

*Table II*
Percentage of labelled cells and mean grain count per labelled cell 8 days after period of labelling with H³-thymidine.

| Blood | | | Skin window II | | |
|---|---|---|---|---|---|
| Cell type | Percent of labelled cells | Grain count per labelled cell | Percent of labelled cells | Grain count per labelled cell | Cell type |
| All mononuclear cells | 15.2 | 12.2 | 1.3 | 4.8 | all mononuclear cells |
| Large mononuclear cells | 8.6 | 7.8 | 1.8 | 5.1 | large mononuclear cells |
| Large lymphocytes | 16.2 | 10.2 | | | |
| Medium-sized lymphocytes | 23.0 | 12.4 | 1.0 | 4.5 | medium-sized mononucl. cells |
| Small lymphocytes | 14.5 | 12.8 | 1 1 | 4.0 | small mononucl. cells |
| Large basophilic mononuclear cells | 0.8 | 3 0 | | | |
| Lympho-monocytes | 9.5 | 5 1 | | | |
| Monocytes | 1 7 | 3 4 | 1.8 | 5 1 | large mononuclear cells macrophages) |
| Granulocytes | 20.6 | 4.5 | 21.5 | 4 5 | granulocytes |

*Table III*
Percentage of labelled cells and mean grain count per labelled cell 24 to 48 h after period of labelling with India ink.

| Blood | | | Skin window III | | |
|---|---|---|---|---|---|
| Cell type | Percent of labelled cells | Grain count per labelled cell | Percent of labelled cells | Grain count per labelled cell | Cell type |
| All mononuclear cells | 11.9 | 15.1 | 2 6 | 6.6 | all mononuclear cells |
| Large mononuclear cells | 23.5 | 16 5 | 2 5 | 6.4 | large mononucl. cells |
| Large lymphocytes | 41.0 | 18.7 | | | |
| Medium-sized lymphocytes | 28.9 | 15.2 | 3.2 | 7.0 | medium-sized mononucl. cells |
| Small lymphocytes | 2.9 | 11.5 | 1 8 | 5 2 | small mononucl. cells |
| Large basophilic mononuclear cells | 0 | 0 | | | |
| Lympho-monocytes | 27.8 | 14.5 | | | |
| Monocytes | 5.4 | 11.6 | 2.5 | 6.4 | large mononuclear cells (macrophages) |
| Granulocytes | 2.0 | 5.0 | 1.2 | 3.0 | granulocytes |

applied skin windows—we found a labelling as shown in Table III *. Large and medium-sized lymphocytes (Fig 3) and in a little less degree, lympho-monocytes have strongly phagocytized carbon particles. Monocytes (Fig 3) and small lymphocytes of the blood and skin window mononuclears (Fig 4) are rarely labelled with carbon grains. The large basophilic mononuclears of the blood never show phagocytized particles. Some kind of correspondence appears to exist between blood monocytes or small lymphocytes on the one hand and skin window cells on the other. All other blood mononuclear cells show quite a different kind of labelling by phagocytosis.

Each individual animal followed the general trend illustrated for the groups.

*Discussion*

According to our studies large mononuclear cells of the blood especially the monocytes, showed a similar labelling as the large mononuclear cells of the skin window: the macrophages. Before one can derive from this similarity an identity of the blood and the skin window mononuclears, the following objections must be rejected.

(1) Contrary to the skin window granulocytes the skin window mononuclears might not originate in the blood but in the local connective tissue. This objection has been refuted by several investigators. CRONKITE *et al* (10) and BRAUNSTEINER *et al* (5) found, that in $H^3$ thymidine labelled rats the connective tissue cells of the subcutis were only rarely (less than 1 %) labelled, while after applying an inflammatory irritation in this tissue 40–100 % of the appearing inflammatory mononuclear cells were labelled without further $H^3$ thymidine having been added. These authors concluded, that all or nearly all inflammatory cells must be derived from blood leucocytes. LEDER and CRESPIN (17) observed in their histological serial studies, that the blood mononuclears emigrated into the inflammatory tissue in large numbers. These authors further stated that as to cytochemistry the skin window macrophages fundamentally differed from the connective tissue cells but showed no difference to the large blood mononuclears. VOLKMAN and GOWANS (24) demonstrated by parabiosis experiments, that labelled pre

* A detailed description of phagocytosis of India ink by the lymphatic system will be published elsewhere (23).

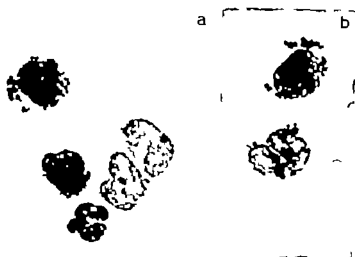



*Fig 3* Peripheral blood of a ra 24 h after an intravenous injection of India ink ( ) On the left two large lymphocytes are labelled th carbon particles On the right two unlabelled monocytes; (b) above carbon-labelled large lymphocyte below carbon-labelled monocyte. Giemsa stain ( 1150)



*Fig 4* Skin window of 26 h, 38 h after the rat had received intravenously India ink. Only one macrophage is labelled with carbon particles Giemsa stain ( 1150)

cursors of the skin window macrophages passed from one connected animal to the other.

(2) The labelling of the skin window cells might occur without being influenced by the labelling of the blood leucocytes. The skin window cells might have received their label *after* emigration from the blood into the blood into the skin window. This conception is not true. According to CRONKITE *et al.* (9) $H^3$-thymidine is only available for about one hour after an intravenous injection. We have applied the skin window cover slips not earlier than two hours after the last injection of $H^3$-thymidine. Moreover DNA is rarely or never synthetized by the mononuclear cells in the exudate of inflammation (10, 5) and mitoses in the skin window are extremely rare (4, 18, 19, 21).

(3) The India ink experiment seems to show that lymphocytes cannot participate in macrophage production, because there are many ink labelled lymphocytes in the blood and very few ink labelled macrophages in the skin window. That might be a wrong conclusion, because the ability of emigration of the lymphocytes having filled themselves up which carbon particles might be restricted, whereas other blood lymphocytes, free of ink, penetrate into the inflamed tissue. This objection can also be rejected. Phagocytizing cells are capable of emigration out of the blood vessels and can immigrate into the inflamed tissue, as could be stated from vital observations (8, 11). The little difference in the percentage and the grain count of the ink-labelled cells between blood monocytes and skin window mononuclears could however be explained by the assumption, that those monocytes, which are only weakly laden or contain no carbon particles, get easier into the skin window than the strongly phagocytizing monocytes.

After the evidence has been obtained, that most mononuclear cells of inflammation originate in the blood, one can conclude from a comparison of the labelling of the blood cells to that of the skin window cells, which cell type of the blood mononuclears has transformed into the skin window mononuclears. As the Tables I–III show, *all blood mononuclears as a whole do not emigrate into the skin window.* Also the complete group of the large mononuclears of the blood cannot, as a whole, form the skin window macrophages, as the above data indicate. It is evident that all lymphatic cells—including the lymphomonocytes—do not play any important role in the development of the skin window macrophages. Only the

monocytes show a close and constant correspondence to the skin window cells, particularly to the macrophages. BRAUNSTEINER *et al.* (5) failed to come to clear conclusions in their labelling experiments on leucocytes and skin window cells, because they did not differentiate the group of blood mononuclears. VOLKMAN and GOWANS (24 25) excluded the group of small lymphocytes as a source of macrophages. But they could not decide, wether the monocytes or another rapidly proliferating cell type transform to macrophages, because they differentiated the blood mononuclear cells only into small lymphocytes, ripe monocytes and a pooled group of all other cells.

The labelling studies do not lead us to a satisfactory conclusion, whether the monocytes alone or together with the large basophilic mononuclears produce the large skin window mononuclears. The morphology disproves the idea, that the basophilic cells take part in the cellular population of the skin window. Neither other authors nor we ourselves could observe cells in any phase of the skin window exudate which showed a similarly prominent cytoplasmic basophilia as the basophilic blood mononuclears did. Only JORKE (14) described rarely occuring basophilic mononuclear cells in the skin windows of patients with a lymphoid reaction.

The fact, that the relatively small group of medium-sized and small skin window mononuclears is a little less labelled than the blood monocytes, can be explained by the participation of few weakly labelled blood or tissue cells besides the monocytes. We suppose together with RIIS (21) and LEDER *et al.* (15) that the medium-sized and small skin window mononuclears are in part large mononuclears, which have rounded themselves to small balls because of a thick layer of exudate.

BRAUNSTEINER *et al.* (5) and we ourselves observed exactly the same behaviour and the same mixture of the cellular inflammatory exudate in rats and in man. Therefore one can suppose that not only in rats but also in man the inflammatory macrophages start from the monocytes. This was proved by findings of our laboratory (3). The intensity of the cellular skin window exudate in patients was solely dependent on the number of the monocytes of the peripheral blood, whereas the number of lymphocytes was apparently of no importance. All patients suffering from HODKIN's disease, which was accompanied by the characteristic lymphopenia and monocytosis, showed the development of numerous macrophages

in the skin window. An opposite example was given by the skin window exudate in patients suffering from lymphatic leukemia. In spite of high lymphocyte counts in the blood the inflammatory response and the production of macrophages were moderate when the number of monocytes in the blood was low.

The cellular reaction in the skin window did not particularly change after an additional antigenic stimulus [egg white: Riis (21); homologous lymphocytes: Wolf-Jürgensen and Schwartz (26); tuberculine: Begemann and v. Zawadsky (3)]. This is important, since Rebuck has suggested that by adding antigen to the skin window lesion the emigration of lymphocytes and the transformation of these into macrophages become more distinct than in the ordinary sterile inflammation.

The development of the inflammatory macrophages from the blood monocytes in rat and man is after these results quite manifest. Thus we have a first insight into the function of the monocyte which has been obscure up to now. After the granulocytes, easily exposed to destruction, the monocytes form the second line of defence against any damage of the tissue. After having defended the tissue as a macrophage the monocyte can later play an important role in the rebuilding of the destroyed tissue. The transformation of the macrophages into fibroblasts and other connective tissue cells seems clearly proved (8, 10, 11, 19).

It remains a matter of dispute, why Braunsteiner *et al.* (4) were able to transfer the delayed type hypersensitivity with skin window cells from sensitized men to unsensitized ones. Since the granulocytes play no part in this type of hypersensitivity, there are only two explanations possible. Either not only the lymphocytes but also the monocytes transfer the delayed hypersensitivity, as Chase (7) has put to discussion, or the few lymphocytes, which find their way into the skin window, are sufficient to transfer the allergy.

We do not maintain that the lymphocytes do not play any role in inflammation and that they are never capable of transformation into large mononuclear cells or even macrophages—we can only affirm, that they do not behave so in the sterile inflammation of the skin.

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## *Summary*

The often discussed problem, whether it is the lymphocyte or the monocyte, which forms the macrophage of inflammation, could be solved This kind of macrophages are transformed monocytes of the blood. In rats the white blood cells were labelled with $H^3$-thymidine or India ink. After that sterile inflammation of the subcutis (modification of Rebuck' skin window) was caused. By comparing the characteristic labelling pattern of the different leucocytes with the labelling of the cells of the inflammatory exudate the identity of monocytes and macrophages could be shown.

## *Zusammenfassung*

Das oft diskutierte Problem, ob die Hautfenstermakrophagen von Lymphocyten oder von Monocyten abstammen, konnte gelöst werden Diese Art von Entzündungsmakrophagen sind umgewandelte Blutmonocyten. Bei Ratten wurden die weißen Blutzellen mit $H^3$ Thymidin oder Tusche markiert. Anschließend wurde eine sterile Entzündung der Subkutis (Modifikation des Rebuck'schen Hautfensters) provoziert. Durch Vergleich des charakteristischen Markierungsmusters der verschiedenen Leukozytentypen mit der Markierung der Entzündungszellen konnte die Identität von Monozyten und Makrophagen gezeigt werden.

## *Résumé*

Le problème souvent discuté de savoir si c'est le lymphocyte ou le monocyte qui forme le macrophage d'inflammation, pu être résolu ces macrophages représentent des monocytes transformés du sang Chez des rats, les leucocytes ont été marqués à l'aide de thymidine tritié ou d encre de chine. Puis une inflammation stérile du tissu cellulaire sous-cutané été provoquée (modification de la fenêtre cutanée de Rebuck). En comparant les modes de marquage des différents leucocytes avec celui des cellules de l'exsudat inflammatoire, l'identité des monocytes et des macrophages pu être démontrée.

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# Sickle Cell Disease and Glucose-6 Phosphate Dehydrogenase

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About two years ago it was suggested that the enzyme deficiency of glucose-6-phosphate dehydrogenase (G-6-PD) was more common in sickle cell disease than in the general population (8). The first published figures indicated 42% of defect in 44 cases of sickle cell disease compared with 14% in 197 normals (11). Subsequently it was found that the incidence of the enzyme defect was 37% in 56 cases of sickle cell haemoglobin C disease and 48% in 100 cases of sickle cell anaemia (6).

These data conflicted with a previous report where it was found that there was an increased incidence of G-6-PD defect in sickle cell trait, sickle cell haemoglobin C disease, but not sickle cell anaemia (14). However the same workers pointed out that there is a marked elevation of the enzyme activity in the red cells of patients with sickle cell anaemia, whether measured by the glutathione stability test (2) or the quantitative assay of G-6-PD (15).

At a later stage, inhibitors of G-6-PD, promazine and dapsone, were used for the treatment and prevention of sickle cell crises. It is interesting to note that it had already been observed that phenothiazines inhibit *in vitro* sickling (3) and that *in vivo* administration of dapsone inhibits *in vitro* sickling (1), at least in individuals with sickle cell trait. In the course of therapeutic studies, further observations were made on G-6-PD activity. These observations form the basis of the present report.

It should be emphasized at the outset that some confusion may be avoided by the use of the term defect to represent the genotype as distinct from the phenotype. Just as one may find the SS haemo-

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globin phenotype in a person with the genotype for AS when thalassaemia trait is also inherited, so it may be possible to find a normal or elevated activity of G-6-PD in a person with the genotype for G-6-PD defect. This anomalous condition is brought about by the fact the enzyme content of young red cells in haemolytic anaemias may be increased several fold (13)

Even in normal females, the presence of heterozygous or partial defect may not lead to measurable diminution of the enzyme level On the other hand, homozygous females and hemizygous males usually show a marked lowering of the enzyme level which is sometimes referred to as complete defect although the reduction may be less than 90%.

For the above mentioned reasons genetic studies have been carried out when feasible. A limited number of quantitative assays have been carried out. Some 250 cases have been investigated in more or less detail and form the basis of this report.

### *Materials and Methods*

This study has been in progress for almost 2 years. Records have been kept of 86 patients who were seen in consultation and 164 who attended the clinic. Only the age, sex, haemoglobin pattern and enzyme activity were recorded for the consultation cases. More detailed studies were made on the clinic cases, some of whom were admitted to the Clinical Investigation Unit for study or treatment.

Haematological studies were carried out by routine methods including the microhaematocrit. Blood was collected from finger or arm vein and preserved with heparin or ethylenediamine tetra-acetic acid.

*Table I*

Proportion of cases with G-6-PD defect according to haemoglobin pattern and sex.

| Pattern | SS | SC | S th | SD | Total | % |
|---|---|---|---|---|---|---|
| Male | 41/95 | 16/50 | 1/4 | 0/0 | 58/149 | 40* |
| Female | 34/62 | 13/27 | 4/5 | 0/1 | 51/95 | 54 |
| Total | 75/157 | 29/77 | 5/9 | 0/1 | 109/244 | 45 |
| Percent | 48 | 39 | | | | |

$\chi^2 = 5.6$, $P = 0.025$

$\chi^2 = 2.0$, $P = 0.157$

G-6-PD activity was estimated by the methaemoglobin reduction test (4) the percentage of methaemoglobin being read visually. Quantitative assays of the enzyme activity were made using modification (5) of the original spectrophotometric method (7). Reduced glutathione was estimated by colorimetric procedure (2).

The haemoglobin patterns were determined by paper electrophoresis at pH 8.6. Sickling was observed in a drop of blood mixed with drop of normal saline on slide. The sealed slide was kept for 24 h at 70 ± 2° F. Red cell survival was estimated with the aid of radio-active chromium (6).

The use of promazine for the treatment of sickle cell crises has been described (10) and long term treatment with promazine and dapsone have been proposed (9). The dose of promazine used for the treatment of crises in adults was 100 mg given three times daily for one week. In long term treatment, 100 mg promazine daily was in some cases supplemented by the administration of 50 mg daily of dapsone.

## *Results*

The over all incidence of G-6-PD defect in sickle cell disease was 45% compared with 14% in normal Ghanaians (11). The proportion of cases showing defect was higher in females than males, 54% compared with 40%. In Table I, the incidence of the defect

*Table II*

Proportion of cases with G-6-PD defect according to haemoglobin pattern and ethnic origin.

| Pattern | SS | SC | Sth | SD | Total | % |
|---|---|---|---|---|---|---|
| Ga+Krobo | 16/44 | 5/25 | 1/2 | 0/0 | 22/71 | 31 |
| Akan+Fanti | 13/28 | 6/14 | 4/6 | 0/0.5 | 23/48.5 | 49* |
| Ewe | 3/8 | 2/4 | 0/0 | 0/0 | 5/12 | |
| Yoruba+Ibo | 2/6 | 0/0 | 0/0 | 0/0 | 2/6 | |
| Northern Ghana | 2/5 | 0/1 | 0/0 | 0/0 | 2/6 | |
| English | 0/0 | 0/0 | 0/0 | 0/0.5 | 0/0.5 | |
| Total | 36/91 | 13/44 | 5/8 | 0/1 | 54/144 | 49* |
| Percent | 40 | 30 | | | | |

$\chi^2 = 2.5$, $P = 0.150$

is analyzed according to haemoglobin pattern and sex. It may be seen that the proportion of cases with defect was higher in sickle cell anaemia than in sickle cell haemoglobin C disease, 48% compared with 39%.

In 144 of the cases a record was made of the ethnic origin. This information is given in Table II which suggests that the incidence of the enzyme defect is slightly higher in the Akan Fanti patients than in the Ga Krobo patients, 49% compared with 36%. The ratio of cases of sickle cell thalassaemia to cases of sickle cell anaemia was also higher in the Akan Fanti group as compared with the Ga Krobo.

An analysis of the incidence of the defect in different age groups is given in Table III. There are no statistically significant differences.

Some 60 patients were treated with promazine at the time of sickle cell crisis. The incidence of G-6-PD defect in these patients

*Table III*

Proportion of cases with G-6-PD defect according to age and haemoglobin pattern or sex.

| Age (years) | SS Sth | SC+SD | Male | Female | Total | % |
|---|---|---|---|---|---|---|
| Under 1 | 2/8 | 3/3 | 2/6 | 3/5 | 5/11 | |
| 1 to 2 | 9/17 | 0/2 | 7/14 | 2/5 | 9/19 | |
| to 4 | 9/17 | 2/5 | 4/12 | 7/10 | 11/22 | 50 |
| 4 to 8 | 21/44 | 3/7 | 13/29 | 11/22 | 24/51 | 47 |
| 8 to 16 | 24/45 | 2/5 | 14/31 | 12/19 | 26/50 | 52 |
| 16 to 20 | 9/18 | 3/9 | 5/12 | 7/15 | 12/27 | 44 |
| 20 or over | 6/15 | 15/49 | 11/45 | 10/19 | 21/64 | 33 |
| Total | 80/164 | 28/80 | 56/149 | 52/95 | 108/244 | 44 |

$\chi^2 = 0.25$ $P = 0.60$

(Table IV) was 30 / compared with 45 / for the group as a whole. The difference was characteristic of sickle cell haemoglobin C disease as well as sickle cell anaemia.

*Table IV*

Proportion of cases with G-6-PD Defect among cases with sickle cell crisis treated with phenothiazine.

| Type of crisis | SS+Sth | SC SD | Total | % |
|---|---|---|---|---|
| Hyperhaemolytic | 0/2 | 0/0 | 0/2 | |
| Haemolytic | 5/14 | 0/0 | 5/14 | |
| Painful | 10/30 | /13 | 12/43 | 28 |
| Hypoplastic | 1/2 | 0/0 | 1/2 | |
| Total | 16/47 | 2/13 | 18/60 | 30* |
| Percent | 36 | | | |

Compared with 45 for entire series, $\chi^2 = 7.3$, $P = 0.007$
Compared with 48 for all SS+Sth, $\chi^2 = 3.7$ $P = 0.020$

*Table V*

Proportion of cases with G-6-PD defect among patients selected for long term treatment.

| Treatment | SS+Sth | SC+SD | Total | |
|---|---|---|---|---|
| Promazine alone | 8/16 | 3/9 | 11/25 | 44 |
| Promazine and dapsone | 17/37 | 3/16 | 20/53 | 38 |
| Total of both groups | 25/53 | 6/25 | 31/78 | 40 |
| Percent | 47 | 24 | 40* | |

Compared with 37 for SC+SD cases, $\chi^2 = 2.3$, $P = 0.132$
Compared with 45 for the entire group, $\chi^2 = 1.0$, $P = 0.320$

Of the cases attending the clinic, 72 were thought to have symptoms of sufficient severity to justify long term administration of promazine (Table V) The incidence of G-6-PD defect in these cases was almost the same as in the series as a whole. During 23 695 patient days of observation on long term treatment, there were 6 instances of crisis of sufficient intensity to lead to hospitalization One of these attacks occurred in a female patient with partial defect (No 736) the other 5 attacks occurred in patients with normal or elevated enzyme activity

Some 20 estimations of G-6-PD activity were carried out by the quantitative method (Table VI) The Brewer test does not detect the extremely high level of enzyme activity which may be present in cases of sickle cell anaemia. On the other hand, the Brewer test detected enzyme abnormality in four patients who showed quantitative levels within the normal range.

Early in the course of these studies it was noted that some patients with normal enzyme activity at the time of crisis, later showed evidence of enzyme deficiency The number of individuals exhibiting this change was greater among those patients that were

*Table VI*
Quantitative test and Brewer test for G-6-PD

| Case | Sex | Hb Pattern | Haematocrit % | Brewer test methgb. % | Quant. test units/g Hb. |
|---|---|---|---|---|---|
| 611 | M | SS | 22 | 0 | 1082 |
| 733 | F | SS | 18 | 0 | 881 |
| 723 | M | SS | 24 | 0 | 815 |
| 581 | M | SS | 31 | 0 | 706 |
| 581 | M | SS | 33 | 0 | 701 |
| 529 | F | SC | 39 | 0 | 606 |
| 529 | F | SC | 39 | 0 | 577 |
| 677 | M | SS | 22 | 0 | 428 |
| 671 | F | SS | 32 | 0 | 421 |
| 713 | M | SS | 20 | 20 | 305 |
| 620 | F | SS | 27 | 100 | 301 |
| 620 | F | SS | 24 | 30 | 250 |
| 640 | F | Sth | 32 | 50 | 205 |
| 627 | M | SS | 21 | 70 | 156 |
| 620 | F | SS | 26 | 70 | 156 |
| 713 | M | SS | 23 | 20 | 136 |
| 713 | M | SS | 24 | 50 | 125 |
| 671 | F | SS | 32 | 20 | 120 |
| 649 | F | Sth | 32 | — | 120 |
| 735 | F | SC | 28 | 80 | 50 |

treated with promazine. Data on 21 patients treated with promazine, that developed positive Brewer tests, are given in Table VII, along with the suspected genotype. The genotype was ascertained on the basis of genetic studies or long term observation while under treatment with promazine. Cases were considered normal when the defect was transient.

The genetic studies are given in Table VIII. It may be seen that there are 4 cases, marked with an asterisk, in which the patient showed evidence of enzyme defect but neither the mother or father in the case of one female, and not the mother in the case of three males showed evidence of enzyme defect. On the other hand there were 11 cases in which both the mother and the patient showed normal enzyme activity and 14 cases in which both the patient and the mother showed enzyme defect.

The level of reduced glutathione was estimated in 27 cases and 24 controls (Table IX). In the controls with normal enzyme activity the average level of reduced glutathione was 71 mg / compared

*Table VII*
Cases in which treatment affected G-6-PD activity

| Case | Sex | Hgb pattern | Initial % methgb. | Subsequent % methgb. | Suspected genotype |
|---|---|---|---|---|---|
| 445 | M | SS | 0 | 20 | normal* |
| 725 | M | SS | 0 | 20 | normal |
| 671 | F | SS | 0 | 20 | heterozygous |
| 715 | M | SS | 0 | 20 | hemizygous |
| 482 | F | SS | 0 | 30 | normal |
| 492 | M | SS | 0 | 30 | normal |
| 567 | M | SS | 0 | 30 | hemizygous |
| 666 | M | SS | 0 | 30 | hemizygous |
| 733 | F | SS | 0 | 20—40 | heterozygous |
| 728 | M | SS | 0 | 40 | normal |
| 649 | F | SCh | 0 | 50 | heterozygous |
| 510 | M | SS | 0 | 10—30 | hemizygous |
| 595 | M | SS | 0 | 40—50 | hemizygous |
| 568 | F | SS | 0 | 60 | heterozygous |
| 698 | M | SS | 0 | 40—60 | hemizygous |
| 677 | M | SS | 0 | 70 | hemizygous |
| 501 | M | SS | 0 | 40—70 | hemizygous |
| 744 | F | SS | 0 | 60—80 | homozygous |
| 620 | F | SS | 0 | 30—100 | homozygous |
| 792 | M | SC | 30 | 100 | hemizygous |
| 600 | F | SS | 50 | 100 | homozygous |

Considered normal when defect was transient.

### *Discussion*

The fact that there is a much higher incidence of G-6-PD defect in cases of sickle cell disease than in the general population seems to be firmly established for the chief ethnic groups in Ghana. The higher incidence of G-6-PD defect in females as compared with males is to be expected in view of the sex linked inheritance with partial dominance. There is no evidence that the correlation between sickle cell disease and G-6-PD defect is due to population segregation.

*Table IX*

Reduced glutathione of red cells according to haemoglobin pattern and G-6-PD activity

| Hgb. pattern | G-6-PD status | Number of cases | % Haematocrit Ave. | SE | Glutathione mg% Ave. | SE |
|---|---|---|---|---|---|---|
| AA+AS | normal | 15 | 43 | 3 | 71 | 4 |
| AA+AS | defect | 9 | 40 | 7 | 47 | 3 |
| SC | normal | 6 | 38 | 2 | 72 | 7 |
| SC | defect | 2 | 36 | 4 | 50 | 9 |
| SC | treated | 6 | 35 | | 61 | 2 |
| SS | normal | 4 | 24 | 3 | 72 | 5 |
| SS | defect | 4 | 23 | 3 | 86 | 2 |
| SS | treated | 5 | 24 | 2 | 76 | 4 |

*Table X*

Size of spleen, foetal haemoglobin level, G-6-PD status and red cell survival in sickle cell anaemia.

| Case | Age years | Sex | Spleen size | Foetal hgb. % | G-6-PD % methgb. | G-6-PD status | Red cell survival days | SE |
|---|---|---|---|---|---|---|---|---|
| 667 | 14 | M | +++ | 10 | 0 | normal | 3.5 | |
| 445 | 16 | M | +++ | 1 | 0 | normal | 4.0 | |
| 725 | 32 | M | ++ | 14 | 0 | normal | 4.5 | |
| 323 | 21 | F | ++ | 10 | 0 | normal | 4.5 | |
| 523 | 21 | F | ++ | 10 | 0 | normal | 5.0 | |
| 555 | 14 | F | +++ | 1 | 0 | normal | 7.0 | |
| Ave. | 20 | — | — | 6 | 0 | normal | 4.6 | 0.5 |
| 677 | 6 | M | ++++ | 20 | 70 | hemiz. | 4.5 | |
| 713 | 10 | M | +++ | 8 | 50 | hemiz. | 5.0 | |
| 510 | 7 | M | 0 | 1 | 50 | hemiz. | 5.5 | |
| 492 | 24 | M | 0 | 1 | 30 | hemiz. | 7.0 | |
| 567 | 13 | M | +++ | 1 | 60 | hemiz. | 7.0 | |
| 666 | 9 | M | 0 | 7 | 30 | hemiz. | 9.0 | |
| 620 | 20 | F | 0 | 11 | 100 | homoz. | 9.0 | |
| Ave. | 13 | — | — | 7 | 56 | defect | 6.7 | 0.8 |

The lack of any significant difference in the incidence of the defect at different age levels in sickle cell disease may be due to the small number of cases in the extreme age groups. It is possible that the presence of G–6–PD defect enhances the liklihood of early diagnosis because of an increased incidence of jaundice.

The relatively low incidence of the defect in cases that were treated for crisis compared with other cases of sickle cell anaemia may be due to a tendency for cases with defect to have fewer crises, or for G-6-PD activity to be higher at the time of crisis. Crises occurred in 5 patients while on long term treatment with promazine and 4 of the 5 patients had normal or elevated enzyme activity.

The quantitative assay for G-6-PD showed good agreement with the Brewer test, except in cases with an intermediate degree of activity when a lower than normal value was found in the Brewer test and a normal value in the quantitative assay. This difference may be a consequence of the fact that the Brewer test expresses enzyme activity in terms of whole blood and the quantitative assay gives activity in terms of units per gram of red cell.

The appearance of G-6-PD defect in cases being treated with promazine might be due to an effect of the drug on the test itself, but the quantitative assay also shows a fall in activity. It is also possible that under promazine therapy there is a rise in the average age of the red cells and this would lead to a decrease in enzyme activity. This hypothesis is compatible with the observation that nucleated red cells tend to diminish during promazine therapy and that there is an increase in the half life of the red cells (6).

The genetic studies support the diagnoses with respect to enzyme activity in all but 4 instances when the mother did not show the expected diminution of enzyme activity. However this occurred in only 4 out of 15 instances when a defect was expected in a female. Other workers have also encountered females with the genotype for partial or heterozygous defect and the phenotype for normal activity.

In patients with sickle cell anaemia there was normal level of free glutathione but stability was not tested.

Theories which might explain the correlation between G-6-PD defect and sickle cell disease include population segregation, superior malarial protection by the combination of abnormalities, a beneficial effect of the enzyme defect on sickle cell disease or visa versa. At first we, like other workers (14) expected that patients

with the enzyme defect and sickle cell disease would be less well off than patients with sickle cell disease and normal enzyme activity Others have shown that there is no disadvantage to the combination (14) and we lean over to the opposite view that the enzyme defect has a favourable effect on the course of sickle cell disease


## *Summary*

The incidence of G-6-PD defect w three times as high in sickle cell disease as in the general population of Ghana. It was higher in females than males, in sickle cell anaemia th in SC disease and in the Akan-Fanti patients as compared with the Ga-Krobo cases. The level of activity tended to be high t the time of crisis and low after promazine therapy There was good agreement between the Brewer and quantitative tests lthough the former wa more sensitive, in cases proven by genetic study Red cell survival w slightly longer in cases with the enzyme defect, which seems to have fa ourable effect on the course of sickle cell disease

## *Zusammenfassung*

Der Mangel an Glukose-6-Phosphatdehydrogenase war bei Patienten mit Sichelzellkr kheit dreimal häufiger als bei der Durchschnittsbevölkerung on Ghana. Die H ufigkeit wa grösser bei Frauen als bei Männern, bei Sichelzellanämie ls bei SC-Krankheit sowie bei Patienten us Akan-Fanti als bei solchen aus Ga-Krobo. Die Ak iv ta war hoch im Zeitpunk einer Krise und niedrig nach Promazinbehandlung Es bestand eine gute Übereinstimmung zwischen dem Brewer Test und der quantitativen Bestimmung, obgleich der erstere empfindlicher war bei Fällen, die durch genetische U tersuchungen gesichert waren. Die Überlebenszeit der Erythrozyten war bei Fällen m Enzymdefekt etwas länger Dieser scheint einen günstigen Effekt uf den Verlauf der Sichelzellkrankheit zu haben.

## *Résumé*

Le manque en G-6-PD était trois fois plus fréquent hez des malades tteints de drépanocytose q en général dans la population du Ghana. Il étai plus f équent chez les femmes que hez les hommes, dans la drépanocytose que dans la maladie SC, ainsi que chez les malades de Akan-Fanti par rapport à ceux de Ga-Krobo. L activité était forte dans les temps de crise et faible après le traitement à la promazine. Le test d Brewer concorda bien ec les tests quantitatifs, tout en étant plus sensible dans les cas certifiés par des études génétiques. La survivance des érythrocytes était quelque peu plus longue dans les cas ayan un manque d enzyme Celui-ci semble avoir un effet favorable sur le décours de la drépanocytose


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# A New Thalassemic Syndrome: Homozygous Hemoglobin S Disease Delta Thalassemia

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The heterogeneity of thalassemia is well known. INGRAM AND STRETTON (4, 5) have distinguished beta and alpha thalassemia according to the genetic involvement of the beta or alpha chain. More recently FESSAS (3) described delta thalassemia as due to a mutation involving only delta chain production. In beta thalassemia, Hb-$A_2$ levels are frequently elevated, whereas in alpha thalassemia Hb-$A_2$ is normal and in delta thalassemia, Hb-$A_2$ values in the heterozygote are approximately 1.5% (normal 2.8±0.4) and in the homozygote Hb-$A_2$ is absent.

Because of the incidence of beta and delta thalassemia in this area, it would seem likely that one should encounter frequent interactions between these genes and the gene for Hb-S. Hb-S beta thalassemia interactions have been described (1, 7), the incidence in this area being approximately 0.1%. The present paper describes the interaction of delta thalassemia with Hb-S.

## Methods

The procedures used for determination of hemoglobin, hematocrit, red blood count, reticulocyte count, osmotic fragility, alkali denaturation, isolation and quantitation of the hemoglobin fractions and isotopic studies are described in a previous publication (8).

The fraction subsequently designated as Hb-S showed decreased solubility in the reduced state; on agar electrophoresis pH 6.2 separated from Hb-A and on hybridization studies was shown to be a beta chain abnormality.

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## *Case History*

A 38 year old colored male was first seen t local hospital in 1953 for anemia and splenomegaly t which time splenectomy was performed. A diagnosis of sickle cell disease was made in 1956 on the basis of positive sickle cell preparation. Since that time, the patient has had frequent crises and bilateral status ulcers. Multiple ttempts skin grafting were unsuccessful.

Both parents of the patient died of old age. Of 13 siblings, 9 are dead, 2 dying of burns, 7 of unknown causes and the remaining three, two sisters and brother are alive and anemic.

The patient was first seen by us on the evening of the 10th of June, 1965, with the complaints of severe neck, and abdominal pain and elevated temperature. Physical ex amination t this time disclosed colored male in obvious distress with blood pressure 120/60 temperature 100 F pulse 80 and weight 140 lb The pertinen physical findings at that time were ( ) hepatomegaly 2 to 3 cm below the right costal margin with lef epigastric cruciat incision and (b) chronic foul smelling ulcers involving both lower legs and almost completely encircling the left leg The skin around the ulcers show considerable depigmentation.

A skeletal X-ray survey showed an osteoporotic trabecular structure with marked sclerotic changes in the pelvis and both hips. There ppeared to have been aseptic necrosis of the left hip. Some cod fishing of the lower thoracic ertebral bodies were also seen and thought chiefly to be arthritic in nature.

While in the hospital, the patien was placed on high protein diet, 25 mg pyridoxine daily 1.2 million units of procaine penicillin every 12 h IM tetracycline 500 mg by mouth every 6 h for fiv days. The leg ulcers were washed daily with hydrogen peroxide and warm saline compresses. The patient was placed on anticoagulant therapy consisting of alternating doses of 5 and 10 mg of Coumadin every other day Six days after admission, granulation tissue appeared in both leg ulcers and the ulcers continued to heal with the patien being discharged 13 days after admission on anticoagulant therapy

Pertinent laboratory findings this time were as follows hemoglobin 8.1 g% hematocrit 24% reticulocytes 9 2 white blood count 24,700 with normal differential. Examination of the red cells disclosed frequen target cells, sickle cells, 1+ hypochromia, 2+ polychromasia, 2+ poikilocytosis, 2+ macrocytosis, 2+ anisocytosis and 1+ microcytosis. Other laboratory findings were Total bilirubin 0 1 mg%, plasma hemoglobin 21 mg% alkaline phosphatase 9 KA units, total serum protein 6.9 g%

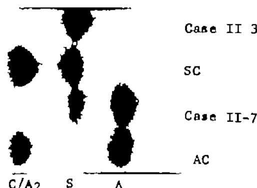



*Fig. 1* Hemoglobin electrophoresis on starch gel pH 8 1 benzidine stain)

the hemoglobin phenotype in case II-3, II 7 and II 9. However for case II-4 to have inherited only the delta chain abnormality she must either be illegitimate or the result of a crossover. Although the former possibility can not be definitely ruled out, it would appear to be unlikely.

In contrast to the severe or moderately severe hemolytic phenomenon found in homozygous Hb-S or Hb-S beta thalassemia, the patient presents a mild hemolytic disorder with no history of jaundice, transfusional therapy and only leg ulcers and bone crises. The finding of a mild hemolytic picture are consistent with those described by Aksoy (1) in sickle cell alpha thalassemia disease.

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## *Summary*

A case of homozygous Hb-S delta thalassemia is presented. The genetic inheritance of the genes is discussed and related to the family pedigree. Observations regarding other genetic combinations are also presented and discussed. It is apparent that this combination presents a mild hemolytic disease.

## *Zusammenfassung*

Es wird ein Fall von homozygoter Hb S-Deltathalassämie beschrieben. Die Vererbung der Gene wird diskutiert und in Beziehung zum Stammbaum gebracht. Beobachtungen anderer genetischer Kombinationen werden erörtert. Diese Kombination scheint eine leichte hämolytische Krankheit hervorzurufen.

## *Résumé*

Un cas homozygote de HbS-delta-thalassémie est décrit. L'hérédité des gènes est discutée et mise en relation avec l'arbre généalogique de la famille. Des observations concernant d'autres combinaisons génétiques sont présentées et discutées. Il est évident que la combinaison présente ne provoque qu'une légère maladie hémolytique.


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# Varia

## European Society of Haematology

### Meeting of Athens, September 10—14 1967

At the loth Congress of the European Society of Haematology in Strasbourg, 1965, it was decided that this Society be merged with the International Society of Haematology Eastern Hemisphere. The International Congresses are to be held every two years only In the intervening years meetings on specific topics are to take place in Europe The present information concerns the first such meeting proposed by the European Society of Haematology

This meeting is organized by the Hellenic Society of Haematology on behalf of the European Society of Haematology President A. GOUTTAS, Secretary-General H. TRIVIDES.

The following subjects have been chosen (1) Inherited abnormalities of the red blood cells; (2) Physiology and pathology of the last phase of coagulation (3) Treatment of malignant blood diseases. The mornings will be devoted mainly to plenary sessions, at which reviews on various aspects of the main subjects will be presented by invited speakers. The afternoons will be reserved for scientific communications related to the three topics, at simultaneous sessions

The meeting will be held the hotel Athens Hilton. A social and ladies program as well as excursions after the meeting will be arranged.

Please direct all correspondence to the secretariat of the meeting /o Prof. Dr A. GOUTTAS, Herodotou 6, *Athens 139* (Greece).

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